THE UNIVERSITY *of* EDINBURGH

Mobilome and antibiotic resistance in *Acinetobacter baumannii*

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2014

I. Abstract

Acinetobacter baumannii is an important microorganism involved in hospital-acquired infections with a remarkable ability to develop resistance to multiple antibiotics (multidrug-resistance, MDR) which makes it a highly troublesome pathogen in many hospitals around the world. Third-generation cephalosporins (such as ceftazidime) and carbapenems (such as imipenem and meropenem) represent important treatment options for infections caused by this microorganism. Nevertheless, the number of strains resistant to these antibiotics has been increasing during the last decade.

The ability to capture, mobilise and regulate the expression of resistance-genes of this microorganism is a cornerstone factor in the development of the MDR, where the Mobilome, defined as "all the mobile genetic elements in a cell", is responsible for its genetic plasticity.

The aim of this work was to analyse the role of insertion sequences (ISs), transposon-like structures, resistance-plasmids and IS*CR1*-like elements in the resistance to carbapenems and ceftazidime in *A. baumannii*. Fifteen carbapanem-resistant strains of *Acinetobacter baumannii* isolated from Chile and two ceftazidime-resistant strains from the United Arab Emirates were studied. Different ceftazidime- and carbapenem-resistance genes were analysed and their genetic environments were characterised.

The Mobilome in the carbapenem-resistant strains was composed of insertion sequences (ISs), specifically by IS*Aba1* associated with $bla_{\text{OXA-51-like}}$, IS*Aba3* associated to $bla_{\text{OXA-58}}$, which in turn was detected in two different plasmids, and IS*Aba15* interrupting IS*Aba3*. In

the case of the ceftazidime-resistant strain, the presence of an IS*CR1* element was harbouring the $bla_{\text{PER-7}}$, which was detected in a megaplasmid.

The Mobilome, in the strains analysed, was composed of a wide variety of genetic elements, such as plasmids, insertion sequences, IS*CR*-like elements, which reflects the ability of *A. baumannii* to use different genetic platforms to capture and use resistance genes, making the Mobilome an important contributor in the resistance and the dissemination of resistance genes among nosocomial pathogens around the world.

II. Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Andrés Felipe Opazo-Capurro.

III. Dedication

This thesis is dedicated to my family, which is my source of strength, perseverance and love; and to my “Edinburgh family”, for your friendship, support and for making my time in Edinburgh the best experience of my life.

IV. Acknowledgments

Firstly, I would like to acknowledge my supervisor Professor Sebastian Amyes whose advice and kindness was very important for the completion of this study. I am very grateful for his confidence and his advice, not only related to the work in the laboratory, but also in making me feel more comfortable and confident with my own work, which will be, undoubtedly, very important for my future career.

I want to thank Dr. Gerardo González-Rocha, who provided the Chilean samples used in this work and for our important discussions and collaborations. I would also like to thank Dr. Tibor Pál who provided the samples from United Arab Emirates and whose collaboration led to the production of an important publication.

I would like to thank Dr. Bruno Lopes for our discussions about *Acinetobacter baumannii* where we shared some ideas and important concepts about techniques. In addition, I would like to thank Norya, Maher, Monem and Leena for their ideas and help in my work and for making the time in the laboratory very pleasant.

Finally, I would like to thank Dr. Catherine Doherty for her help, which was decisive in developing the project, and I want to thank the Chilean National Commission for Scientific and Technological Research (CONICYT) for funding the scholarship that allowed me to do this PhD in Edinburgh.

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VI. Publications and presentations.

- **Opazo AF**, Lopes B, Sonnevend A, Pal T, Ghazawi A, Amyes SGB. Ceftazidime Resistance in *Acinetobacter baumannii* from the United Arab Emirates. (Oral, 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, 2011, Chicago).
- **Opazo A**, Lopes B, González-Rocha G, Amyes SGB. Detection of IS*26* in carbapenem non-susceptible *Acinetobacter baumannii*. (Poster, 9TH International Symposium on the Biology of *Acinetobacter*, 2013, Cologne)
- Lopes BS, Gould IM, **Opazo AF**, Amyes SGB. The resistance profile of *Acinetobacter baumannii* from the Aberdeen Royal Infirmary. *International Journal of Antimicrobial Agents*. 2012; 39: 361-362
- **Opazo A**, Sonnevend A, Lopes B, Hamouda A, Ghazawi A, Pal T, Amyes SGB. Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates. *Journal of Antimicrobial Chemotherapy*. 2012; 67: 1619-22.
- **Opazo A**, Dominguez M, Bello H, Amyes SGB, González-Rocha G. OXA-type carbapenemases in *Acinetobacter baumannii* in South America. *Journal of Infection in Developing Countries*. 2012; 6: 311 – 316.
- **Opazo A,** Vali L, Al Obaid K, Dashti A, Amyes SGB. Novel genetic structure harbouring bla_{PER-1} in ceftazidime-resistant *Acinetobacter baumannii* isolated from Kuwait. *International Journal of Antimicrobial Agents*. 2014; ahead of publication.

VII. Abbreviations

ADC	*Acinetobacter*-derived cephalosporinases
AMC	Amoxicillin/clavulanic acid
ATM	Aztreonam
bp	Base pairs
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Blood-stream infections
CAZ	Ceftazidime
CLSI	Clinical and Laboratory Standards Institute
CIP	Ciprofloxacin
cm	Centimetres
CN	Gentamicin
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
EC	European clone
ESAC	Extended-spectrum AmpC
ESBL	Extended-spectrum β-lactamase
FDA	US Food and Drug Administration
FEP	Cefepime
h	hours
ICU	Intensive care unit
ICUn	Intensive care unit (neurology)
ICUs	Intensive care unit (surgical)
InCU	Intermediate care unit
IPM	Imipenem
IST	Isosensitest
ITU	Intensive therapy unit
kb	Kilo-bases pairs
kDa	Kilo-Dalton
L	Litre
M	Molar
MDR	Multidrug-resistant
MEM	Meropenem
MIC	Minimum inhibitory concentration

min	Minute
mg	Milligram
mL	Millilitre
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center of Biotechnology Information
NCTC	National Collection of Type Cultures
OMP	Outer membrane protein
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PG	Peptidoglycan
pM	Pico-moles
RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
s	Seconds
TAE	Tris/acetate/ethylenediaminetetraacetic acid
TBE	Tris/borate/ethylenediaminetetraacetic acid
TGC	Tigecycline
tRNA	Transfer RNA
TZP	Piperacillin/tazobactam
U	Units
V	Volts
VAP	Ventilator-acquired pneumonia
VRE	Vancomicin-resistant *Enterococci*
w/v	Weight/volume
µL	Microlitre
µM	Micromolar

VIII. Tables and Figures.

IX. Contents

Chapter 1 Introduction

1.1. A short history of antibiotics

1.1.1. The pre-penicillin era.

Antibiotics are defined, in modern terms, as "any synthetic or naturally occurring low molecular weight molecule that inhibits bacterial growth" (Gillings, 2013). The history of antibiotics starts back in 1400s with the use of bismuth and mercury in the treatment of syphilis caused by *Treponema pallidum* (Shlaes, 2010). These compounds are harmful to people but, in proper doses, they are more toxic to the disease-causing microorganism. The "modern era" of antibiotics started in the beginning of the 20th century, when Paul Ehrlich introduced an arsenical-compound, arsphenamine, for the treatment of diseases caused by microbes. This new antibiotic compound, also known as Salvarasan, was successful for the treatment of microbial infections and thanks to its high effectiveness, was called the "Ehrlich's magic bullet" (Bosch and Rosich, 2008; Shlaes, 2010). Due to his discovery, Ehrlich was awarded with the Nobel Prize of Medicine in 1908. However, the usage of this drug was restricted as it is also toxic for humans as it is an arsenic-based compound. It was necessary to develop new innocuous compounds able to attack only the infectious microorganisms. Later, in the 1930's, sulphonamides were identified as new options for bacterial infections. The first drug of this new group, Prontosil, was synthetized by Bayer chemists (Domagk, 1957). This drug was a prodrug, which after be metabolised produces its active form, sulfanilamide (reviewed by Shlaes, 2010). As in the case of "Ehrlich's magic bullets", Gerhard Domagk, who was responsible of the characterisation of the antimicrobial capacity of Prontosil, won the Nobel Prize of Medicine in 1939. Mainly due to their low manufacturing costs, sulfonamides were produced in large quantities and used widely to treat bacterial infections (Aminov, 2010). To illustrate the impact of sulfonamides in public

health, a study carried out in 1938 in a hospital from Chicago USA; it showed that the mortality among patients with pneumonia, treated with Dagenan (sulphonamide) ranged between 6 to 17.6% while in the untreated patients group, the mortality rates oscillated between 23 to 50% (reviewed by Shlaes, 2010). However, these drugs were effective against a limited number of bacterial pathogens and had no effect on several infection-producing microorganisms (reviewed by Guilfoile, 2007).

1.1.2. The antibiotic revolution

In 1928, Sir Alexander Fleming and his group made one of the most important contributions in the history of modern medicine. He and his colleagues studied the antibiotic properties of *Penicillium notatum*, a mould able to produce a molecule which inhibits the bacterial growth. This compound had a stronger activity than sulfonamides and affected a broader spectrum of microorganism (Fleming, 1929). It was not until 1940 that a chemistry group led by Ernest Chain and Howard Florey purified the antibiotic compound, called penicillin and by 1945 they were able to produce large quantities of this new drug (Ligon, 2004). The first use of penicillin in England was in 1941. A patient with a serious infection caused by *Staphylococcus aureus*, previously treated with sulfonamides, was then treated with a limited amount of penicillin, and the infection was controlled (reviewed by Guilfoile, 2007). Following this success, penicillin usage increased widely and the improvement of the chemical processes to find and synthesize antibiotics enabled the discovery of a number of new agents. The 1945-1960 period is known as the "golden era" of antibiotics, where most of the classes of antibiotics, currently in clinical use, were initially characterised (Wright, 2007).

The following decade, between 1970-1980, was a period where a considerable number of new antibiotics were produced using the natural scaffold of the previously describe

antibiotics, in order to overcome bacterial resistance and increased their effectiveness. The manufacture of these semi-synthetic antibiotics led to this period being known as the "golden age of antibiotic medicinal chemistry" (Wright, 2007). Nonetheless, after this period, the number of new antibiotics approved for clinical use decreased alarmingly, mainly due to the rapid appearance of resistant bacteria. Only a few new antibiotics were approved in the late 1990's and early 2000's, such as linezolid, tigecycline and daptomycin, resulting in reduction of therapeutic options to treat microbial infections. In this sense, Sir Alexander Fleming, during his Nobel lecture in 1945, expressed a major concern about the misuse of antibiotics, saying that "there may be a danger in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body" (Alexander Fleming, Penicillin, Nobel Lecture, December 11, 1945). This message was the first warning of a phenomenon that is affecting us worldwide today.

1.2. Types of antibiotics

The antibiotics can be characterised according to their chemical structures and mechanism of action.

1.2.1. Structural classes.

1.2.1.1. β-lactams

More than half of the antibiotics available today and some currently in development are β-lactams; this is due to their high effectiveness and safety (Nicolau, 2008). The principal structural feature of these drugs is the presence of the β-lactam ring, also known as "chemical warhead" (Guilfoile, 2007). Figure 1.1 shows the structure of penicillins, the first members of this group.

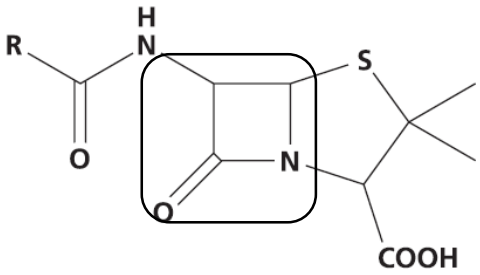

Figure 1. General structure of penicillins. The β-lactam ring is highlighted in the black box (adapted from -Guilfoile, 2007). R – side chain.

There are four main subgroups of β-lactams including penicillins, cephalosporins, monobactams and carbapenems (Guilfoile, 2007).

Penicillins

This subgroup comprises the original drug, called Penicillin G or benzylpenicillin and its derivatives including ampicillin, amoxicillin and methicillin (Miller, 2002). Penicillin G is still currently widely used. The penicillin-derivate are antimicrobials generated by chemical modifications of the side chain (R- as represented in Figure 1) on the basic nucleus of penicillin (Wright and Wilkowske, 1987). The aim of the modification of the side chain is to increase their antibacterial activity, resist enzymatic hydrolysis (penicinillases) and improve their pharmacokinetic properties (Sköld, 2011).

Cephalosporins

The cephalosporins subgroup are closely related to penicillin, but they were extracted from a different microorganism, *Cephalosporium acremonium*. The first member of this subgroup, cephalosporin C, was discovered in 1948 by Giuseppe Brotzu when he studied the ability of this microorganism to inhibit the growth of *S. aureus* (reviewed by Bo, 2000).

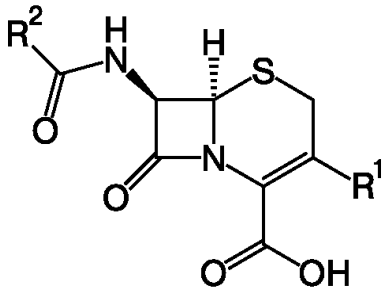

Figure 2. Chemical structure of cephalosporins. R^1 – side chain 1; R^2 – side chain 2.

The cephalosporins differ from penicillin by possessing a six-membered heterocyclic ring, containing a sulphur atom (dihydrothiazine ring) attached to the β-lactam structural ring (Figure 2). The variations in the chain sides (R^1 and R^2 in Figure 2) increase their antimicrobial activity, compared with penicillins, and provide them longer half-lives in the

host (Kalman and Barriere, 1990). Currently there are five generations of cephalosporins based to some extent on the year of development but also on their spectrum of activity (Kollef, 2009).

First generation cephalosporins are effective against Gram-positive and Gram-negative bacteria but poorly effective against anaerobic microbes while second-generation cephalosporins have a broader-antibacterial activity including, specifically in the case of cefoxitin, activity against anaerobic pathogens (reviewed by Kalman and Barriere, 1990). Third-generation cephalosporins are very effective against Enterobacteriaceae and also, in the case of ceftazidime and cefoperazone, they are very active against *P. aeruginosa*. Whereas fourth-generation cephalosporins (cefepime and cefpirome) are more resistant to β-lactamases and their action spectrum includes methicillin-resistant *Staphylococcus aureus* (MRSA), largely because of their rapid penetration (Bijie *et al.*, 2005). Ceftopribole and ceftaroline, which comprise the fifth generation of cephalosporins, are resistant to β-lactamases produced by *S. aureus* and have a high affinity for the PBP 2a of MRSA, representing a useful alternative for treating infections caused by this multidrug-resistant pathogen (Duplessis and Crum-Cianflone, 2011; Kisgen and Whitney, 2008).

Monobactams

This subgroup of β-lactams comprises a unique member, aztreonam (Duma, 1988). This compound was produced by a soil bacterium, *Chromobacterium violaceum* (Johnson and Cunha, 1995). The β-lactam ring is not attached to an extra ring (monocyclic β-lactam) unlike cephalosporins and penicillins (Figure 3). This is the simplest β-lactam with antibacterial activity.

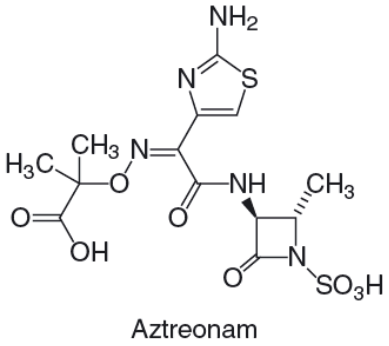

Figure 3. Chemical structure of aztreonam (Sköld, 2011).

Aztreonam is only effective against aerobic Gram-negative bacilli so it must be administrated in conjunction with other antibiotics in many cases (Guay and Koskoletos, 1985). Monobactams do have an important characteristic; they do not induce allergic reactions in penicillin-allergic patients (Johnson and Cunha, 1995), representing a helpful alternative to treat patients with this condition.

Carbapenems

This last subgroup of β-lactams are derived from thienamycin, produced by a soil microorganism, named *Streptomyces cattleya* (Kahan *et al*., 1979). As with other members of the β-lactam group, these drugs are formed around a β-lactam ring, as shown in Figure 4. They differ from cephalosporins in that the β-lactam ring is fused to a five-member thiazolidine ring. The differences with penicillins are the substitution of the sulphur atom, in the five member ring, for a carbon atom and the presence of an unsaturated bond in the thiazolidine ring, between C2 and C3 (Nicolau, 2008).

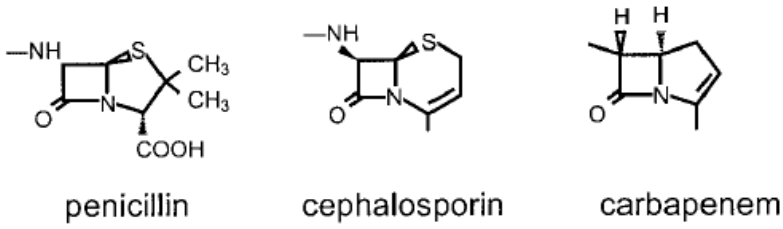

Figure 4. Comparison between the chemical structures of β-lactams (Knapp and English, 2001).

Structurally, the carbapenems differ from each other by their side chain (Figure 5). The first carbapenem, imipenem, was approved in 1987. However, this drug was degraded by the enzyme dehydropeptidase (DHP-1), present in the renal tubules (reviewed by Zhanel *et al*., 2007). To avoid this degradation, imipenem is supplied with cilastatin, a compound that inhibits DHP-1 (Norrby, 1995). Meropenem, the second member of the subgroup, was approved in 1996. This antimicrobial differs from imipenem in that it was not inhibited by DHP-1 (Norrby, 1995) and could also be given to children. These antimicrobial agents possess a broad spectrum of activity, affecting mainly Gram-negative, but also some, Gram-positive pathogens and also are effective in the treatment of nosocomial (hospital-acquired) infections (Zhanel *et al*., 2007). Meropenem differs from imipenem in possessing a pyrrolinidyl group at the position 2 which may be the reason of its higher activity against Gram-negative pathogens in comparison with imipenem (Zhanel *et al*., 2005).

Ertapenem, approved in 2001, is an antimicrobial drug used to treat complicated infections not involving nosocomial pathogens, such as *Enterococcus* spp. and non-fermentative Gram-negative pathogens (Livermore *et al*., 2003). Doripenem was approved in 2012 by the US Food and Drug Administraion (FDA), for the treatment of adults with complicated intra-abdominal infections and complicated urinary tract infections including pyelonephritis (Rai

et al., 2013). The broad spectrum of activity is associated to the stability against hydrolytic degradation (Zhanel *et al*., 2007).

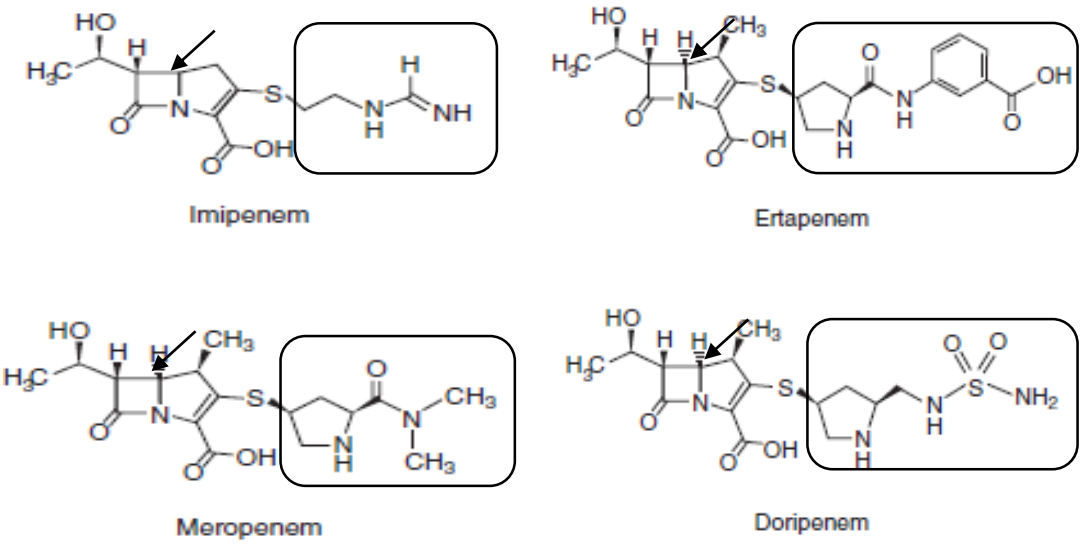

Figure 5. Chemical structure of carbapenems. The side chains are highlighted in the black boxes (adapted from Zhanel *et al*., 2007). The arrows show the carbon 6 of the chemical structure of carbapenems.

This stability is due to the presence of a *trans*-α-1-hydroxyethyl substituent at the 6 position, which is exclusive when compared with cephalosporins and penicillins, where there is a *cis* configuration (Moellering *et al*., 1989). Due to the characteristics described above, these antimicrobial drugs represent an important alternative to the treatment of serious infections and to the elimination of resistant-bacteria.

1.2.1.2. Tetracyclines

Tetracyclines are antimicrobial drugs produced by many species members of the *Streptomyces* genera (Duggar, 1948). Tetracycline, oxytetracycline, doxycycline and

minocycline are the main members of this group (Griffin *et al.*, 2010). The name refers to their chemical structure that is composed by a four-ring core carrying different functional members; for instance, the oxytetracycline, produced by *S. rimosus*, has a hydroxyl group in the third ring (Figure 6) instead a hydrogen atom as in tetracycline.

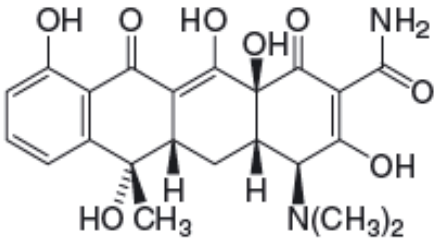

Figure 6. Chemical structure of oxytetracycline (Sköld, 2011).

Tetracyclines are very effective against many different pathogens, hence are classified as broad-spectrum drugs that are also effective against mycoplasma, intracellular chlamydiae, rickettsiae and some protozoan parasites (Nelson and Levy, 2011).

1.2.1.3. Aminoglycosides

The first aminoglycoside, streptomycin, was isolated by Selman Waksman in 1943 from *Streptomyces griseus* (Raju, 1999), representing the third clinically useful natural antibiotic discovered after penicillin. In the beginning, streptomycin was widely used against *Mycobacterium tuberculosis,* depicting the first antibiotic used to treat tuberculosis (Sköld, 2011). The chemical structure of aminoglycosides, illustrated in Figure 7, shows that the molecule is composed of two or more amino sugars (aminoglyco) fused to an amino-cyclitol nucleus (Begg and Barclay, 1995).

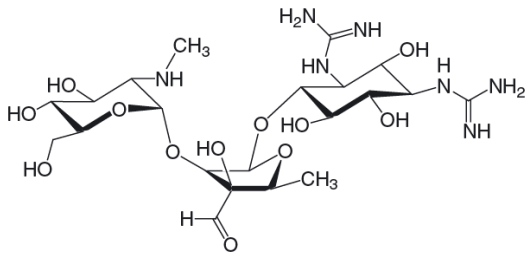

Figure 7. Chemical structure of streptomycin (adapted from Skold, 2011).

Later in 1949, neomycin was isolated from *Streptomyces fradiae* followed by kanamycin, which was isolated, in 1957, from *Streptomyces kanamyceticus* (Begg and Barclay, 1995). Afterwards, another important member of this group, gentamicin, was obtained from *Micromonospora purpurea* (Weinstein *et al*., 1963). Netilmicin, tobramycin and amikacin were introduced after 1976 (Begg and Barclay, 1995). Tobramycin, netilmicin, gentamicin and amikacin are currently being used in clinical practice while streptomycin is not generally used because of its side effects, such as nephrotoxicity and ototoxicity (Begg and Barclay, 1995; Sköld, 2011). They are several factors favouring aminoglycoside use, for example synergism with β-lactams, clinical effectiveness and rapid concentration-dependent bactericidal effect (Begg and Barclay, 1995).

1.2.1.4. Macrolides

The first member of this group used clinically, erythromycin (Figure 8), was discovered in 1952 and was produced by *Streptomyces erythreus*. It consisted of a large lactone ring (macro) with two attached sugar substituents (Douthwaite and Champney, 2001). The members of this group possess a complex chemical structure formed by a macro-lactone ring that varies in size from 12 to 16 atoms.

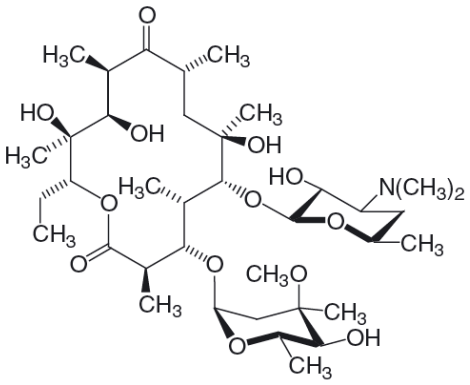

Figure 8. Chemical structure of erythromycin (Sköld, 2011).

Erythromycin is effective against Gram-positive cocci, such as *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *S. aureus*, and also possesses activity against *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydia pneumoniae* (Shah, 1998). Due to some limitations of erythromycin, such as poor oral bioavailability, a short half-life in the host, side effects, and drug-drug interactions, new macrolides were designed in order to improve pharmacokinetic properties (reviewed by Zhanel *et al*., 2001). These new macrolides members, such as clarithromycin and azithromycin, have better pharmacokinetic properties and higher antimicrobial activity (Carbon, 1995). However, the number of resistant-bacteria has increased rapidly, decreasing the use of macrolides. In order to improve their activity against this macrolide-resistant-bacteria, a new group of macrolides

was developed, named ketolides, that possess a *keto* group in their 14-membered lactone ring and, clinically, ketolides differ from macrolides in that they do not induce macrolide resistance in *S. pneumoniae* (Bryskier, 1998).

1.2.1.5. Quinolones

In 1962, the first quinolone antibiotic, nalidixic acid, was introduced into medical practice. Nalidixic acid is a synthetic drug that is active mainly against Gram-negative microbes (Oliphant and Green, 2002). After the introduction of nalidixic acid, several antibiotics derived from it were designed, such as gatifloxacin, levofloxacin and ciprofloxacin, which are useful for treating urinary infections caused by Gram-negative enterobacteria (Sköld, 2011). The chemical structure of quinolones comprises two rings, with a nitrogen in position 1, a carbonyl group in position 4 and a carboxyl member in position 3 (Figure 9).

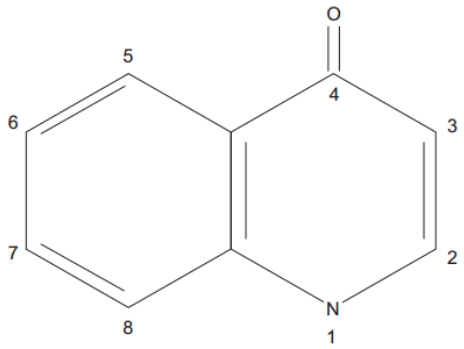

Figure 9. Chemical structure of quinolones (Alós, 2009).

Quinolones are divided in generations according to their spectrum of activity (Naber and Adam, 1998). The first generation quinolones, comprising nalidixic acid and pipemidic acid, have activity mainly against *Enterobacteriaceae*. Since the second generation, a fluoride atom was included in the structure of quinolones, in the position 6, enabling them to be called fluoroquinolones (Blondeau, 2004). In general terms, fluoroquinolones (such as ciprofloxacin, levofloxacin and gatifloxacin) have a higher activity compared with first

generation quinolones, representing important agents to treat infections caused by Gram-negative, Gram-positive and atypical pathogens (Blondeau, 2004).

1.2.1.6. Sulfonamides

These drugs represent the first group of truly selective antibacterial agents approved for medical use. Sulfonamides were introduced in 1935. The selectivity of sulfonamides is due to the fact they inhibit a bacterial enzyme, dihydropteroate synthetase, which involved in important metabolic pathway in bacteria but it does not exist in mammalian cells (McCullough and Maren, 1973). The name is because the presence of a sulphur atom in their structure (sulpha) and an "amide" group in the chemical scaffold (Figure 10).

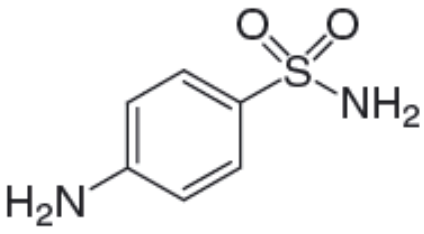

Figure 10. Chemical structure of sulfonamide (Sköld, 2011)

Because they are easy and cheap to synthesize, sulfonamides were extensively used decades ago but their use is very limited nowadays. This decline has mainly been due to the appearance of more effective drugs, the emergence of bacterial resistance and their considerable side effects, such as allergic reactions including Steven-Johnson syndrome (Sköld, 2011).

1.2.1.7. Trimethoprim

Trimethoprim was first used for the treatment of infections in humans in 1962 (Huovinen *et al*., 1995). This drug, administrated alone, was used as prophylaxis for urinary tract infections in Finland, in 1972; when it was introduced to treat patients with acute urinary infections in 1979 (Huovinen, 2001). This drug is related to sulfonamides in the sense that it interferes the same metabolic pathway in bacteria (Sköld, 2011). According to its chemical structure (Figure 11), trimethoprim corresponds to a structural analog of the folic acid pterin moiety, thereby inhibiting the enzyme dihydrofolate reductase in bacteria. The selective action of trimethoprim, affecting only to the bacterial cells, allows its clinical use at low concentrations (Sköld, 2011).

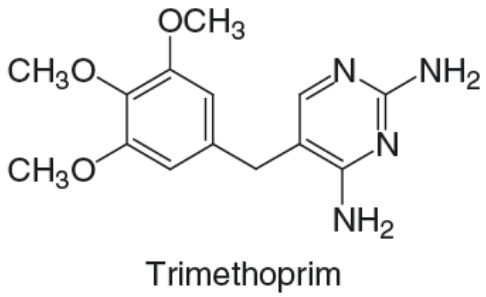

Figure 11. Chemical structure of trimethoprim (Sköld, 2011).

Since trimethoprim and sulfonamides, specifically sulfamethoxazole, attack successive steps in the same metabolic pathway, they have been used in combination, being known as cotrimoxazole (Sköld, 2011). This drug combination was originally a first-line option for the treatment of urinary tract infections, sinusitis and acute bronchitis (reviewed by Strevel *et al.*, 2006), but the toxicity of the sulphonamide component means that the combination is now rarely used in the manority of countries. However, it is sometimes used as a prophylaxis method to prevent opportunistic infections in patients with HIV/AIDS, such as infections

caused by *Pneumocystis jiroveci* (Manyando *et al*., 2013) and nocardiosis (Eshraghi *et al*., 2014).

1.2.1.8. Others

Chloramphenicol was introduced in the 1950s. This drug was the first broad-spectrum bacteriostatic antibiotic affecting both Gram-negative and Gram-positive pathogens (Wareham and Wilson, 2002). This drug is able to cross through the blood-brain barrier ensuring that it is an important drug for treating meningitis (van Niekerk *et al*., 1980; Sköld, 2011). Nevertheless, it is used principally for external purposes, as skin unguent and eye drops; and it is not use for the systemic treatment of bacterial infections because its side effects, blood dyscrasias, which can cause irreversible and fatal aplastic anaemia (Sköld, 2011).

Rifampicin, a member of the rifamycin family with antibacterial activity, was introduced in 1967. This compound does not share any chemical similarity with other families of antibiotics (Furesz, 1970). Rifampicin has a broad spectrum activity, although Gram-negative rods, such as *E. coli*, are less susceptible due to the poor ability of the large rifampicin molecule to penetrate the lipopolysaccharide (LPS) barrier (Sköld, 2011). Its use is almost exclusively to treat infections caused by *M. tuberculosis* (Guilfoile, 2007); although because its low toxicity, its use has been increasing as a combination antimicrobial therapy for the treatment of various infections, including infections caused by *S. aureus, S. pneumoniae, Legionella* spp., *Rhodococcus* spp. and infections caused by fungus (reviewed by Forrest and Tamura, 2010). Interestingly, in a murine model of *A. baumannii* pneumonia caused by susceptible, intermediate or resistant strains, rifampicin, as a monotherapy, was more effective in reducing microorganisms in the infection site than imipenem, sulbactam, tobramycin and colistin (Pachón-Ibáñez *et al*., 2006). However, the usage of rifampicin as a

monotherapy, should be managed under strict control because it induces the rapid appearance of resistant strains both *in vitro* and *in vivo* (Garnacho-Montero and Amaya-Villar, 2010). Additionally, rifampicin could be used in combination with other antibiotics in order to treat infections caused by MDR-*A. baumannii*; for example, rifampicin in combination with polymyxins, such as colistin, has been used to treat infections caused by nosocomial MDR strains (Bassetti *et al*., 2008). Similarly, rifampicin in combination with imipenem or sulbactam showed high efficacy against MDR *A. baumannii* in experimental models of meningitis and pneumonia (Pachón-Ibáñez *et al.*, 2010).

Another important group of antibiotics comprise the polymyxins. Currently there are two polymyxins available for clinical usage, colistin (polymixin E) and polymixin B (Landman *et al*., 2008). Colistin was discovered in 1949 and is produced by *Bacillus polymyxa* subspecies *colistinus* Koyama (Falagas and Kasiakou, 2005). Polymyxins have been used worldwide in topical otic treatments and as ear drops for decades (Falagas and Kasiakou, 2005). These drugs have bactericidal activity against a wide number of Gram-negative pathogens, such as *Escherichia coli*, *P. aeruginosa*, *Acinetobacter* spp., etc. (Landman *et al*., 2008). The usage of polymyxins was stopped during the next decade following its introduction, owing to their toxicity, specifically nephrotoxicity (Kwa *et al*., 2008). However, the use of polymyxins, specifically colistin, has risen during the last decade mainly because the appearance of multidrug resistant MDR-pathogens (Landman *et al*., 2008). According to a work published by Jimenez *et al.,* a case of meningitis caused by a MDR-*A. baumannii* strain was treated by administration of colistin every 6 h, which stopped the infection (Jiménez-Mejías *et al*., 2002). Additionally, in order to treat MDR pathogens, colistin can be co-administrated with other antibiotics. For example, in the treatment of infections caused by MDR-pathogens in patients with cystic fibrosis, the administration of colistin with an antipseudomonal agents, such as ceftazidime or ciprofloxacin, is more effective than the treatment with colistin alone (Conway *et al*., 1997; Falagas and Kasiakou, 2005). As mentioned before, the combination

of colistin and rifampicin has a synergistic bactericidal activity against MDR-pathogens. Additionally, it has been demonstrated that the combination of colistin and ceftazidime is active against MDR *P. aeruginosa in vitro* (Gunderson *et al*., 2003) as the combination of colistin, rifampicin and amikacin has been used for the treatment of infections caused by MDR *P. aeruginosa* in an immunocompromised patient (Tascini *et al*., 2000). In the case of polymyxin B, it has a lower activity against *P. aeruginosa*, *Salmonella* spp. and *Shigella* spp. than colistin (Falagas and Kasiakou, 2005).

Glycopeptides represents another important group of antimicrobial agents. These antibiotics are active against Gram-positive bacteria but they do not have activity against Gram negative pathogens (Colabella and Chagan, 2008). Specifically, relative to other antimicrobials, glycopeptides are larger molecules, which prevents their transfer through the porins in the outer membrane of Gram-negative bacteria; thus making them inactive against this group of microorganisms (Nicolosi *et al*., 2010). Their principal use is to treat infections caused by Gram-positive cocci, such as infections caused by methicillin-resistant *S. aureus* (MRSA); and against anaerobic pathogens, such as the toxin-producing *Clostridium difficile* (Sköld, 2011).

Finally, an important last-generation group of antibiotics corresponds to glycylcyclines, represented by tigecycline (Olson *et al*., 2006). Tigecycline, approved for clinical use in 2005, is a derivative of minocycline (tetracycline-group member) that possess a high activity against pathogens that are hard to treat, such as MSRA, vancomycin-resistant enterococci (VRE), *Acinetobacter baumannii* and extended-spectrum β-lactamase (ESBL)-positive pathogens (Noskin, 2005). Compared with polymyxins, tigecycline is less toxic and, in most patient populations, it is not necessary to adjust the dosage (Stein and Craig, 2006). Due to these features, tigecycline has the potential to be used in several clinical areas. The chemical scaffold of tigecycline (Figure 12) includes the classical structure of tetracyclines, where a *t*-butylglycylamido substituent is located at position 9.

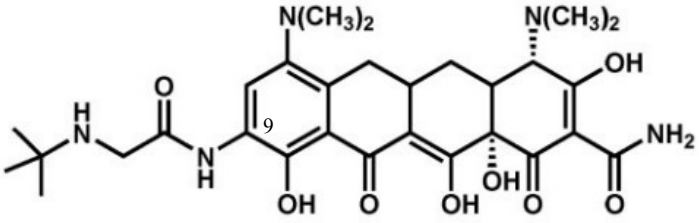

Figure 12. Chemical structure of tigecycline. The 9-*t*-butylglycylamido is indicated in the figure (adapted from Olson *et al*., 2006).

Tigecycline has been approved for treating complicated intra-abdominal infections, complicated skin infections and adult complicated-pneumonia (Stein and Babinchak, 2013). This new-generation drug is still under study in order to analyse where and when it should be used, considering both effectiveness and emergence of resistance (Stein and Babinchak, 2013).

1.2.2. Mechanisms of action

Antibiotics have two different effects in bacteria, either slowing growth (bacteriostatic) or killing the bacteria (bactericidal) and they can be classified according to their mode of action against bacteria. Accordingly, as shown in the Figure 13, there are five main mechanisms of action: (i) inhibition of protein synthesis, (ii) interruption of the cell wall synthesis, (iii) inhibition of an important metabolic pathway, (iv) interruption of nucleic acids synthesis and (v) destabilisation of cellular membranes.

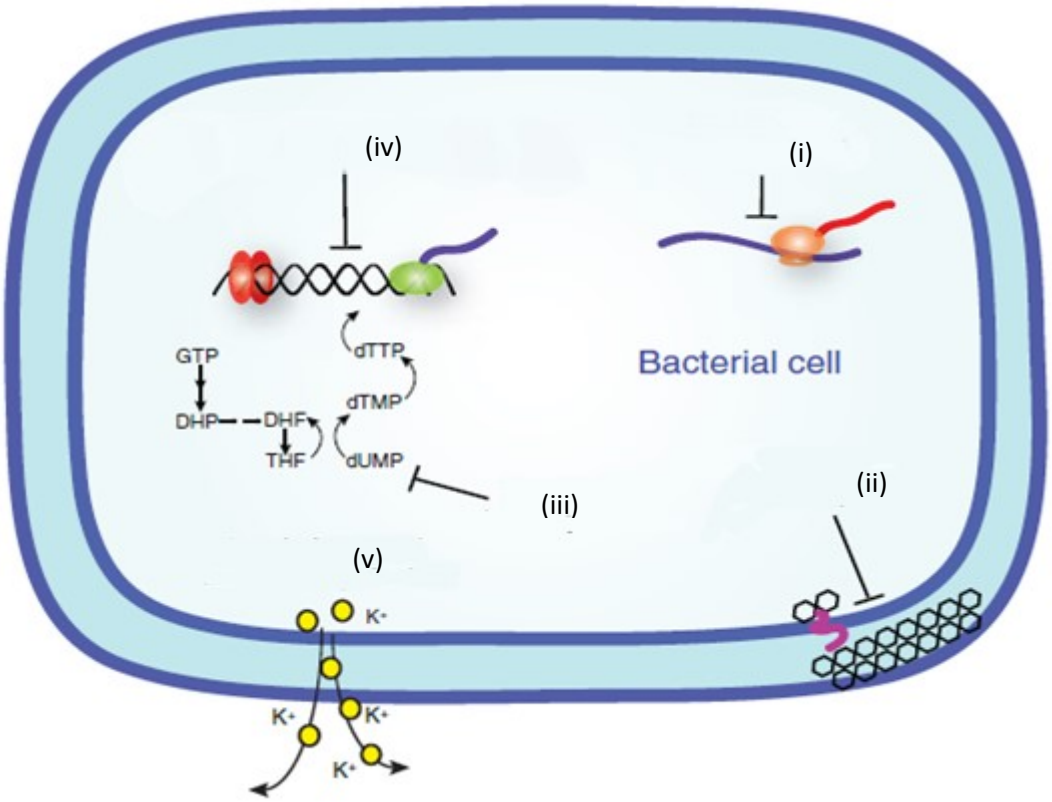

Figure 13. Schematic summary of the mechanisms of action of antibiotics. (i) Inhibition of protein synthesis, (ii) interruption of the cell wall synthesis, (iii) inhibition of an important metabolic pathway, (iv) interruption of nucleic acids synthesis, (v) destabilisation of cellular membranes (adapted from Clatworthy, Pierson, & Hung, 2007).

1.2.2.1. Inhibition of protein synthesis

Living and growing, among others functions, requires the use and supply of new proteins. In both type of cells, prokaryotic and eukaryotic, the synthesis of proteins, called translation, is a process in which the ribosomes are the subcellular structure responsible for carrying out this function. Nevertheless, prokaryotic and eukaryotic ribosomes are sufficiently different to allow antibiotics to effectively attack the bacterial (reviewed by Guilfoile, 2007). In bacteria the ribosome is a nucleoprotein complex, where two-thirds of them comprise RNA and one-third protein. They are called 30S (small) and 50S (large) subunits (reviewed by Walsh, 2003). In turn, the small subunit contains about 20 proteins and a 16S rRNA while the large subunit is formed by 30 proteins, a 23S rRNA and a 5S rRNA (reviewed by Walsh, 2003). Macrolides, such as erythromycin, binds selectively and reversely to the bacterial ribosomes, thus providing bacteriostatic activity (reviewed by Sköld, 2011). The erythromycin interacts with the 23S rRNA of the large subunit, blocking the polypeptide translation, interrupting the production of mature proteins (Schlünzen *et al*., 2001). Tetracyclines and glycylcyclines are bacteriostatic drugs that bind to the 30S ribosomal subunit, specifically to the 16S subunit, blocking the binding of the incoming aminoacyl-tRNAs (Tritton, 1977). Glycylcyclines have a higher affinity than tetracyclines, presumably because they differ in their binding orientation with the 16S subunit (Olson *et al*., 2006). Aminoglycosides represent another group of antibiotics that works by interrupting the biosynthesis of proteins in bacteria. Their activity is based on their ability to bind to the 16S rRNA of the 30S subunit (Moazed and Noller, 1987). Interestingly, unlike the other antibiotics that affect the protein biosynthesis, the aminoglycosides possess bactericidal activity as they induce alterations on the bacterial cells, such as sodium and potassium leakage, and later also to large biomolecules, finally leading to bacterial death (reviewed by Sköld, 2011). Chloramphenicol, a bacteriostatic drug, acts interfering with the synthesis of proteins by binding to the 50S subunit of the bacterial

ribosome (reviewed by Sköld, 2011). A summary of the main antibiotics that affect the production of proteins is shown in Figure 14.

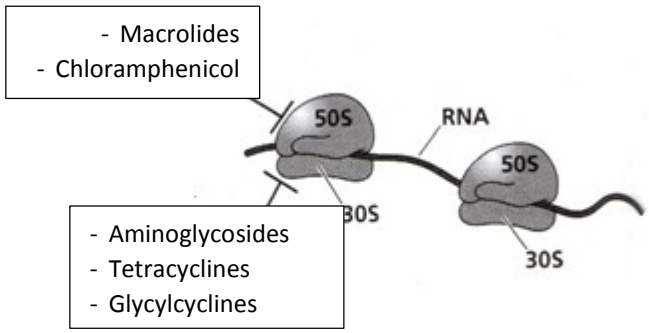

Figure 14. Antibiotics that block the synthesis of proteins (adapted from Walsh, 2003).

1.2.2.2. Interruption of the cell wall synthesis

Both Gram-negative and Gram-positive bacteria possess a peptidoglycan (PG) layer as a component of their cell wall that confers resistance against osmotic lysis. This bacterial wall (Figure 15) is composed of a scaffold of long polysaccharide chains, which are cross-linked between themselves by peptides (reviewed by Sköld, 2011). To create the PG layer, there has to be enzymatic crosslinking of the glycan strands (transglycosylation) and of the peptide strands (transpeptidation) (reviewed by Walsh, 2003). In the enzymatic transpeptidation, catalysed by enzymes called penicillin-binding proteins (PBPs), so-called because they are the target of penicillin and other β-lactams, a pentapeptide attached to a monosaccharide containing a diaminoamino acid with two D-alanines is transferred out through the cell membrane. Afterwards, the monosaccharide unit is aggregated in a growing chain of polysaccharide of the cell wall where the carboxyl member of the peptide can form a peptide bond with the diaminoamino acid added peptide of an adjacent polysaccharide chain, thereby producing a covalent link between two polysaccharide chains (reviewed by Sköld, 2011). The β-lactams drugs inhibit the transpeptidation reaction by binding to the PBPs as there is structural similarity between the β-lactam ring and the D-alanyl-D-alanine dipeptide.

Blocking this important process of the microorganisms makes a newly produced cell wall unstable, leading to lysis of the bacterial cell (reviewed by Walsh, 2003; Sköld, 2011).

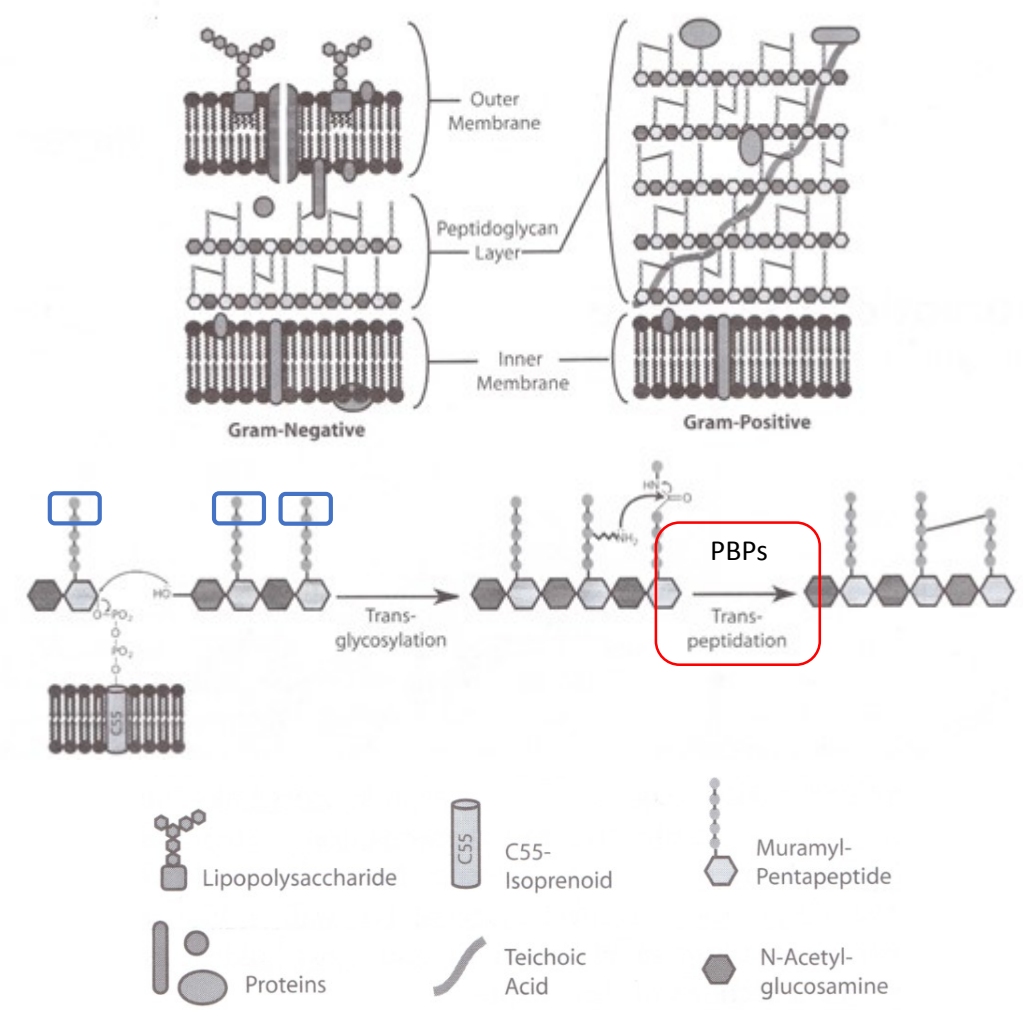

Figure 15. Schematic representation of the structure and biosynthesis of the peptidoglycan in bacteria (modified from Walsh, 2003). PBPs: Penicillin-binding proteins. The target site of β-lactams agents is highlighted in red. The target of glycopeptides, D-Ala$_4$-D-Ala$_5$, is highlighted in blue.

Another group of antibiotics that affects the biosynthesis of the cell wall are the glycopeptides. These antibiotic drugs cannot penetrate through the outer membrane of Gram-negative, thus they are effective only against Gram-positive pathogens (Van Bambeke, 2006). The mechanism of action does not involve inactivation of key enzymes of the cell wall biosynthesis, but functions by binding to units that form the PG at the D-Ala$_4$-D-Ala$_5$ tails (Figure 15) (reviewed by Walsh, 2003). This binding of glycopeptides leads to the interruption of the crosslinking reactions finally producing cell wall lability and bacterial lysis (Kahne *et al*., 2005).

1.2.2.3. Inhibition of an important metabolic pathway

Folic acid is an important coenzyme that is essential for many biochemical reactions which occur inside all the cells. Differentially, bacteria have the ability to synthetize their own folic acid while humans need to get this vitamin from their diet (reviewed by Guilfoile, 2007). This difference permits the use of certain drugs that exclusively affect the pathway of folic acid synthesis of bacteria, avoiding any damage to human cells. The sulfonamides represent a group of drugs which interrupt the biosynthesis of folic acid in bacteria. Sulfonamides, currently represented by sulfamethoxazole, is administrated with trimethoprim, in a combination called co-trimoxazole (reviewed by Sköld, 2011). They are still widely used because they are cheap and effective (Ho and Juurlink, 2011). These drugs act synergistically at different levels in the same pathway, where sulfamethoxazole inhibits the enzyme dihydropteroate synthase (DHPS) in the folate synthesis process while trimethoprim blocks the dihydrofolate reductase (DHFR), which is the enzyme responsible for providing the pyrimidine thymilydate for DNA synthesis (Figure 16). The dihydropteroate is produced assembling GTP and *p*-aminobenzoate (PABA). As sulfonamide is a structural analogue of PABA, it interrupts this step in the specific pathway (Figure 16) (reviewed by Walsh, 2003).

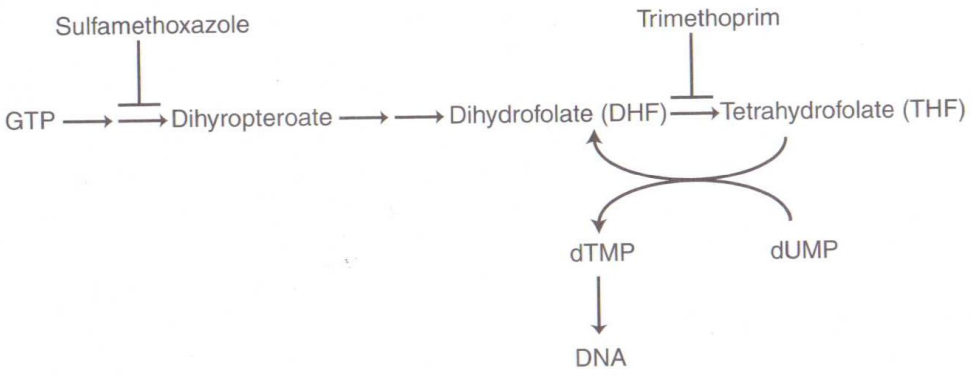

Figure 16. Schematic representation of the bacterial biosynthetic pathway of the folic acid (modified from Walsh, 2003).

Trimethoprim prevents the enzymatic conversion of dihydrofolate (DHF) into tetrahydrofolate (THF). This inhibition occurs because trimethoprim is a structural analogue of the folic acid pterin motif and thus produces competitive inhibition of the DHFR enzyme (reviewed by Guilfoile, 2007; Sköld, 2011). Despite their effectiveness and selectivity, the use of co-trimoxazole has been threatened by the increase in resistant-pathogens during recent years and by the toxicity of the sulphomanide component (reviewed by Walsh, 2003; Sköld, 2011).

1.2.2.4. Interruption of the nucleic acids synthesis

The replication of nucleic acids (DNA and RNA) are key processes necessary for living and growing, both in humans and bacteria. This action makes them an ideal target to inhibit pathogens. The replication of DNA is carried out by a group of enzymes, called topoisomerases, of which there are four members in bacteria. The different processes that occur during the replication and repair of the bacterial DNA are facilitated by the action of

these topoisomerases (Andriole, 2005). The functions of each topoisomerase in bacteria are different, topoisomerase I is responsible for relaxing negative supercoils, topoisomerase II (also called DNA gyrase) introduces negative supercoils and relaxes positive supercoils, topoisomerase III decatenates replication intermediates and bind dimers of DNA, and topoisomerase IV decatenates DNA and eliminates positive and negative supercoils (Pan and Fisher, 1997; Walsh, 2003). In general terms, the mechanism of action of fluoroquinolones is by affecting the changes in the conformation of the DNA necessary for the normal cell functioning. The quinolones act by promoting degradation of the bacterial DNA by binding to the enzyme-DNA complexes of DNA gyrase and topoisomerase IV, leading to the death of the cell (reviewed by Hooper, 1999). In general, the activity of quinolones correlates with inhibition of DNA gyrase in Gram-negative (Figure 17) and with blocking the topoisomerase IV enzyme in Gram positive microorganisms (reviewed by Oliphant & Green, 2002).

Rifampicin is an antibiotic which acts in a different nucleic acid than quinolones (Figure 17). This antibiotic corresponds to a RNA polymerase inhibitor, hence affecting bacterial growth. The effect of rifampicin is due to its ability to bind to the RNA polymerase of prokaryotic organisms, which is a large and complex enzyme formed by five peptide subunits (reviewed by Sköld, 2011). This structure is composed by a core tetramer of αββ'γ subunits and a σ subunit which is dissociable and that is in charge of direct the core polymerase to transcribe specific genes (reviewed by Walsh, 2003). Rifampicin binds to the β subunit in an allosteric site blocking the elongating RNA chain, producing a final inhibition of the bacterial transcription process (Campbell *et al.*, 2001). The corresponding mammalian RNA polymerase does not bind rifampicin, which allows it to act selectively (reviewed by Sköld, 2011).

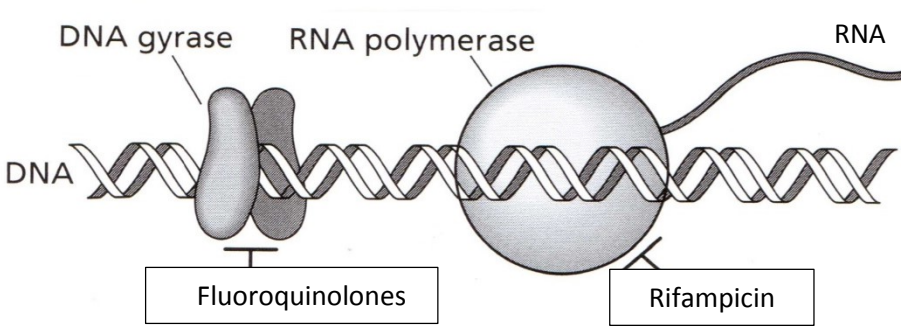

Figure 17. Antibiotics that block DNA and RNA replication (adapted from Walsh, 2003).

1.2.2.5. Destabilisation of cellular membranes

Polypeptide antibiotics, such as polymixin B and polymixin E (colistin), were available for use in the 1960s but their usage was stopped owing their nephrotoxicity. However, due to the dramatic increase of outbreaks caused by MDR-Gram-negative pathogens worldwide, their use has been reconsidered as an option for treating infections caused by these microorganisms (Kwa *et al*., 2008). Polymixin B and colistin are pentacationic molecules that have a bactericidal activity; the variation between them is just one aminoacid (Figure 18). Their activity is due to their chemical nature which allows them to act as outer-membrane-inserting cationic hydrophobic polypeptides (reviewed by Walsh, 2003). Their activity is similar to detergents, increasing the permeability of the cell membranes, leading to cell death (Kwa *et al*., 2008). This mechanism of action involved a first step of electrostatic binding between the cationic polymixin and the anionic LPS of the Gram-negative bacteria, leading to the distortion of the cell membrane (reviewed by Falagas & Kasiakou, 2005). This disturbance is due to polymyxins displace magnesium (Mg^{+2}) and calcium (Ca^{+2}) which are the responsible for stabilizing the LPS macromolecule (Kwa *et al*., 2008). The final result of this process is the increase of the membrane permeability, leading to the leakage of

intracellular content and, consistently, cell lysis (Schindler and Osborn, 1979). However, as mentioned before, these molecules are toxic for humans. Polymyxins are toxic to humans as they affect the renal system (nephrotoxic). There have been some advances in this field, making some modifications in the structure of colistin, trying to avoiding their noxious effects. One example corresponds to polymixin B nonapeptide (PMBN) that lacks the fatty acid tail. This change ensured that PMBN is 15 times less toxic than polymixin B, when was tested in mice (reviewed by Vaara, 2010). Because of this important side effect, it is compulsory to evaluate their use considering both risks and benefits for the patient. Further studies in order to understand the mechanism of nephrotoxicity and focus in decrease this side effects, are necessary.

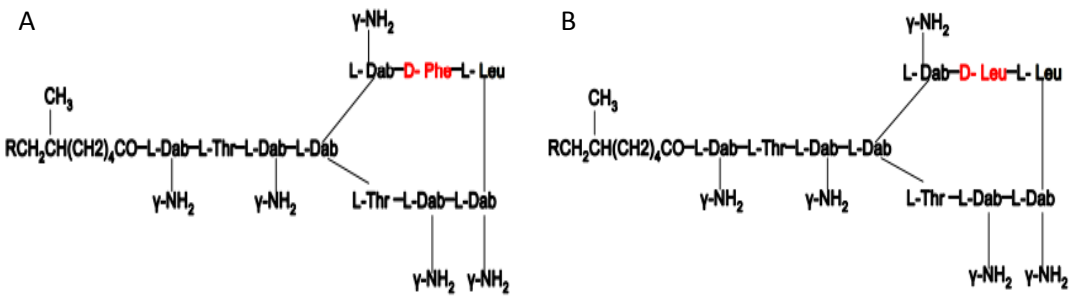

Figure 18. Chemical structure of polymyxins. A: Polymyxins B; B: Colistin. The different aminoacids are highlighted in red (adapted from Kwa *et al*., 2008)**.**

1.3. Mechanisms of antibiotic resistance

Throughout history, whenever a new antibacterial drug is introduced, clinically significant resistance appears. Antibiotic-resistant bacteria can be selected in a hospital environment much faster than in the community, owing to the fact that in the hospitals there is an intensive and constant exposure to antibiotics. This exerts a selective pressure leading to the survival of those bacteria which had acquired the resistance mechanism(s) to face this pressure (reviewed by Walsh, 2003). The three major mechanisms of resistance in clinically important bacteria are: (i) enzymatic inactivation, (ii) efflux and impermeability, and (iii) replacement or modification of the antibiotic target.

1.3.1. Enzymatic inactivation

This mechanism of resistance involves the production of enzymes which can either destroy or modify antibiotics. This is the most important and widely spread mechanism of resistance, specifically in β-lactams-resistance (Bush and Mobashery, 1998).

1.3.1.1. β-lactamases

The hydrolysis mediated by β-lactamases corresponds to the most important mechanism of resistance to β-lactams (Palzkill, 2013). These enzymes hydrolyse the four-member β-lactam ring, producing an inactive β-lactam derivative (Bush, 1989). They are traditionally classified according either by their functional features or their primary molecular structure. According to their amino acid sequences, there are four main classes of β-lactamases, of which classes A, C and D are active-site serine enzymes (Bush & Mobashery, 1998; Bush & Jacoby, 2010). Briefly, the serine acts as a reactive nucleophile, attacking the β-lactam ring and producing an acyl-enzyme intermediate and then a water molecule completes the

hydrolysis reaction, releasing the intact serine-β-lactamase and an inert β-lactam derivative (Figure 19) (Massova and Mobashery, 1998; Walsh, 2003).

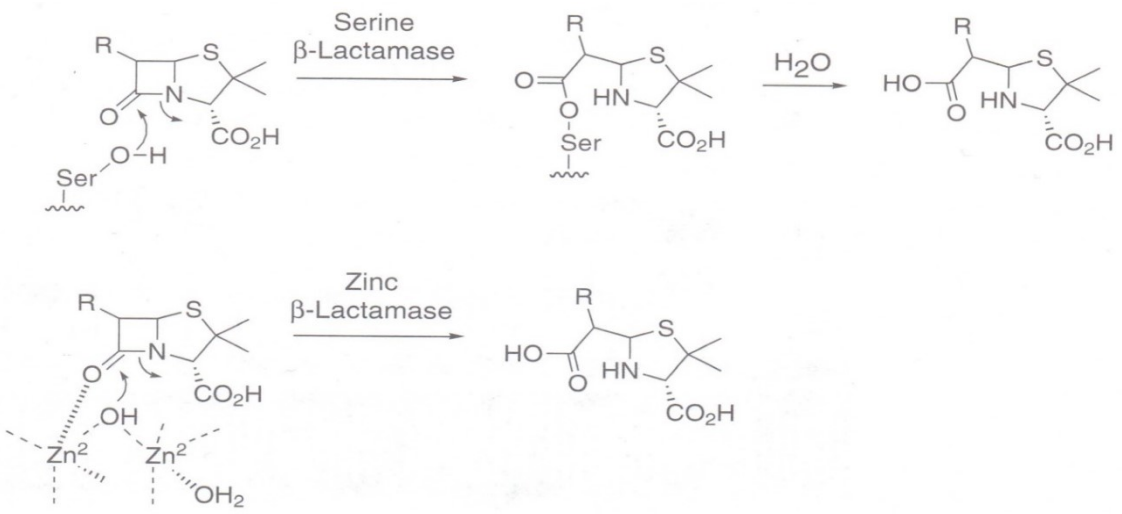

Figure 19. Enzymatic inactivation of β-lactams by serine- and zinc-β-lactamases (adapted from Walsh, 2003).

In the case of the class B β-lactamases, they have a zinc ion in their active site, thus they are called metallo-β-lactamases (MBLs) (reviewed by Walsh, 2003). The MBLs use zinc to activate a water molecule and catalyse its incorporation to the β-lactam ring, producing an inactive product (Palzkill, 2013). The functional classification of β-lactamases is based on their catalytic properties which gives the chance to relate them to their clinical impact. A summary of the classification of β-lactamases is shown in Table 1.

1.3.1.2. Aminoglycoside modifying enzymes.

Aminoglycosides represent another group of antibiotics that are inactivated through two main mechanisms of resistance: enzymatic modifications and decrease in aminoglycosides uptake and accumulation. Unlike β-lactamases, aminoglycoside-resistance is mediated by

modifications in their chemical structure by the addition of different kind of substituents, interrupting their antibacterial activity by blocking the binding between the antibiotic and the ribosomes (reviewed by Walsh, 2003). There are three families of aminoglycoside modifying enzymes (AMEs). The inactivation of aminoglycosides can be achieved through the activity of kinases such as *O*-phosphotransferases (APHs), *O*-adenyltransferases (ANTs) and *N*-acetyltransferases (AACs) that are present in both Gram-negative and Gram-positive pathogens (reviewed by Wright, 1999). The APHs enzymes catalyse the addition of a phosphate group to the molecule of aminoglycoside, while the ANTs kinases incorporate an acetyl member to the chemical scaffold and finally, the ANTs enzymes add an AMP group to the molecule of aminoglycoside (reviewed by Walsh, 2003; Wright, 1999). These enzymatic-modifications lead to produce chemical modified drugs, which bind poorly to ribosomes and where the uptake of aminoglycosides also fails, leading to high level resistance (Mingeot-Leclercq *et al*., 1999).

Table 1. Classification of β-lactamases. A: Classification according to Bush & Jacoby, 2009; B: Classification proposed by Bush, Jacoby & Medeiros, 1995; NI: not included (Table modified from Bush, 2010).

Functional classification (2009)[A]	Functional classification (1995)[B]	Molecular class (subclass)	Common name	Substrates	Examples
1	1	C	Cephalosporinase	Cephalosporins	AmpC
1e	NI	C	Cephalosporinase	Cephalosporins	CMY-37
2a	2a	A	Penicillinase	Penicillins	PC1
2b	2b	A	Cephalosporinase	Penicillins, early cephalosporins	TEM-1
2be	2be	A	Cephalosporinase	Penicillins, cephalosporins and monobactams	SHV-2
2br	2br	A	Inhibitor-resistant penicillinase	Penicillins	TEM-30
2ber	NI	A	Cephalosporinase	Extended-spectrum cephalosporins, monobactams	TEM-50
2c	2c	A	Carbenicillinase	Penicillins, carbenicillin	CARB-3
2ce	NI	A	Carbenicillinase	Cefepime, carbenicillin	RTG-4
2d	2d	D	Cloxacillinase	Penicillins, including cloxacillin and oxacillin	OXA-10
2de	NI	D	Cephalosporinase	Extended-spectrum cephalosporins	OXA-11
2df	NI	D	Carbapenemase	Carbapenems	OXA-23
2e	2e	A	Cephalosporinase	Cephalosporins	CepA
2f	2f	A	Carbapenemase	All β-lactams currently available	KPC-2
3a	3	B (B1, B3)	Metallo-β-lactamase	All β-lactams excluding monobactams	VIM-1
3b	3	B (B2)	Metallo-β-lactamase	Preferential hydrolysis of carbapenems	CphA
NI	4	Unknown			

1.3.2. Efflux and impermeability

This mechanism represents the second major route of antibiotic resistance. The active efflux is mediated by transmembrane proteins, which act like pumps that export antibiotics, often against a gradient of concentration (reviewed by Walsh, 2003). By bioinformatic analysis, there are four main families of efflux pumps described that can extrude antibiotics. The first three families are drug-protons antiporters classified as part of the major facilitator subfamily (MFS), the small multidrug regulator (SMR) family or the RND (resistance/nodulation/cell division) superfamily (reviewed by Van Bambeke *et al.*, 2000). The MFS subfamily members can extrude amphiphilic substrates (mono- or dicationic molecules), including antibiotics such as tetracyclines, fluoroquinolones, rifampicin, chloramphenicol and aminoglycosides (reviewed by Van Bambeke *et al*., 2000; Walsh, 2003). The SMR family includes small proteins of 12-kDa. They can expel lipophilic and multicationic substrates such as tetracyclines and erythromycin. The last proton-dependant group, the RND superfamily, can extrude a wide range of substrates, such as tetracyclines, glycylcyclines, fluoroquinolones, erythromycin, rifampicin, β-lactams, chloramphenicol and aminoglycosides (reviewed by Van Bambeke *et al*., 2000). The second major class of efflux pumps, the ABC family, are ATP-dependent systems. They can eliminate molecules from the intracellular environment through hydrolysis of ATP, thus is a mechanism that needs energy (reviewed by Walsh, 2003). Their substrates include tetracycline, fluoroquinolones, macrolides, rifampicin, chloramphenicol and aminoglycosides (reviewed by Van Bambeke *et al*., 2000). Besides efflux mechanisms, pathogens can modify the expression of some specific porins in the cellular membrane(s), preventing the uptake of noxious compounds. In the case of aminoglycosides, besides the activity of the AMEs, another mechanism of resistance is represented by impermeability, specifically by reduced aminoglycosides uptake. In this case, the reduction of the uptake of these drugs can confer resistance in Enterobacteriaceae and other Gram-negative microorganisms. In some cases, the reduced

uptake may reflect loss of respiratory chain components, leading to reduced bacterial growth and confers low-level of resistance, producing isolates of low clinical impact (Taber *et al*., 1987; Acosta *et al*., 2000). However, other members of the cell envelope, different from the respiratory chain components, may be involved in the decreased uptake of aminoglycosides, such as the olipeptide-binding protein (OppA). This protein is the periplasmic component of a major oligopeptide permease system, which could participate in the transport of aminoglycosides in *E. coli* K-12 (Acosta *et al*., 2000). Specifically, kanamycin-resistant *E. coli* K-12 mutants express low levels of OppA (Acosta *et al*., 2000); whereas *E. coli* K-12 mutant, which produces higher levels of this protein, are more susceptible to aminoglycosides (Kashiwagi *et al*., 1992). Nevertheless, the role of OppA in the resistance to aminoglycosides is controversial as, according to Nakamatsu *et al.*, the OppA protein and the Opp system do not have a direct role in the aminoglycoside uptake and sensitivity in *E. coli* K-12. This can be explained by mutations that affect different components of the bacterial physiology, specifically those that affect the intracellular polyamines pool which control, at the posttranscriptional level, OppA expression and then the activity of aminoglycosides in *E. coli* K-12 (Nakamatsu *et al*., 2007).

Another example is constituted by carbapenem-resistance in *A. baumannii*. In this case, it is well known that carbapenems enter the periplasmic space through a porin called CarO. In the case of some carbapenem-resistant strains, they lack this protein, blocking the entry of the antibiotics and impeding their binding to the target proteins (Mussi *et al*., 2007).

1.3.3. Replacement or modification of the antibiotic target

These mechanisms of resistance are represented by the ability of drug-resistant bacteria to alter the antibiotic target to insensitivity while also preserving its essential cellular function. In the case of methicillin resistance in *S. aureus*, the pathogenic bacteria are not resistant to

methicillin through the production of a β-lactamase but are resistant by the acquisition of the *mecA* gene, which encodes a new PBP, called PBP2a, that is a bifunctional transglycosylase/transpeptidase (Hiramatsu *et al*., 2001; Song *et al*., 1987). β-lactams have a low binding affinity for this PBP2a making *S. aureus* resistant to all kinds of β-lactams (reviewed by Walsh, 2003). Another mechanism of antibiotic-resistance that belongs to this classification group is vancomicin-resistance. There are two main pathogens which share the same mechanism of resistance to vancomycin, *S. aureus* and enterococci (Hughes, 2003). This mechanism involves the change of the acyl-D-Ala_4-D-Ala_5 end of the precursor of PG by acyl-D-Ala_4-D-$lactate_5$. This change in the structure of the precursor of the PG produces a decrease of 1000-fold in the affinity of vancomycin for its target (reviewed by Reynolds and Courvalin, 2005).

In some macrolide resistance, mutations in the DNA gyrase gene leads to the methylation of the 23S rRNA preventing the binding of macrolides to their target (reviewed by Guilfoile, 2007). On the other hand, cotrimoxazole resistance is mediated with the bypass of the normal metabolic pathway; a mutation in a plasmid-encoded gene of DHFR is responsible for producing a new variant of this enzyme, which confers resistance to trimethoprim (Amyes and Smith, 1974). Sulfamethoxazole resistance is achieved by mutations in the *dhps* gene that leads to the generation of a new variant of the dihydropteroate synthase (DHPS) that confers resistance to sulfamethoxazole (Amyes and Smith, 1974). Finally, the mechanism of resistance to polymyxins implies the modification of the structure of the cell wall via reduction of the LPS. Specifically, changes in the lipid A of the LPS controlled by PmrA/PmrB regulatory system, leads to modifications in the charge of the LPS, impeding the attachment of polymyxins to this net (Moskowitz *et al*., 2004).

1.4. Genetic basis of the antibiotic resistance

Antibiotic-resistance can either be innate or be an acquired characteristic. The innate resistance to an antimicrobial is due to the natural characteristics of a specific microorganism, such as the intrinsic resistance to vancomycin in *E. coli*. However, the acquisition of antibiotic-resistance occurs by different genetic mechanisms that allow the spread of genes that confer this phenotype. The mechanisms of resistance acquisition include mutations and capturing of exogenous genes (Giedraitienė *et al*., 2011; Hawkey, 1998). A summary of the genetic mechanisms involved in the acquisition of antibiotic resistance is shown in Figure 20.

The most common mutations are mainly related to the alteration of the target molecules, such as in the case of the quinolone-resistance, where the mutations interfere with the binding of the antibiotic to the ribosome, blocking their antibiotic activity (Nikaido, 2009). Besides alteration of target molecules, mutations can contribute to antibiotic resistance through alterations of the promoter regions of antibiotic-resistance genes or affecting the genes that encode regulatory proteins of these determinants (Martinez and Baquero, 2000). An example of this effect of mutations is represented by the hyperproduction of AmpC β-lactamase in *E. coli*, which is caused by promoter mutation that increases the transcription level of the *ampC* gene (Caroff *et al*., 2000).

On the other hand, the acquisition of resistance genes represents an important feature associated with bacteria, which can allow them to capture and express genes that permit them to survive under antibiotic pressure. The capture of resistance genes can be mediated by three different mechanisms of horizontal gene transfer (HGT): conjugation, transformation and virus-mediated transduction. (Figure 20).

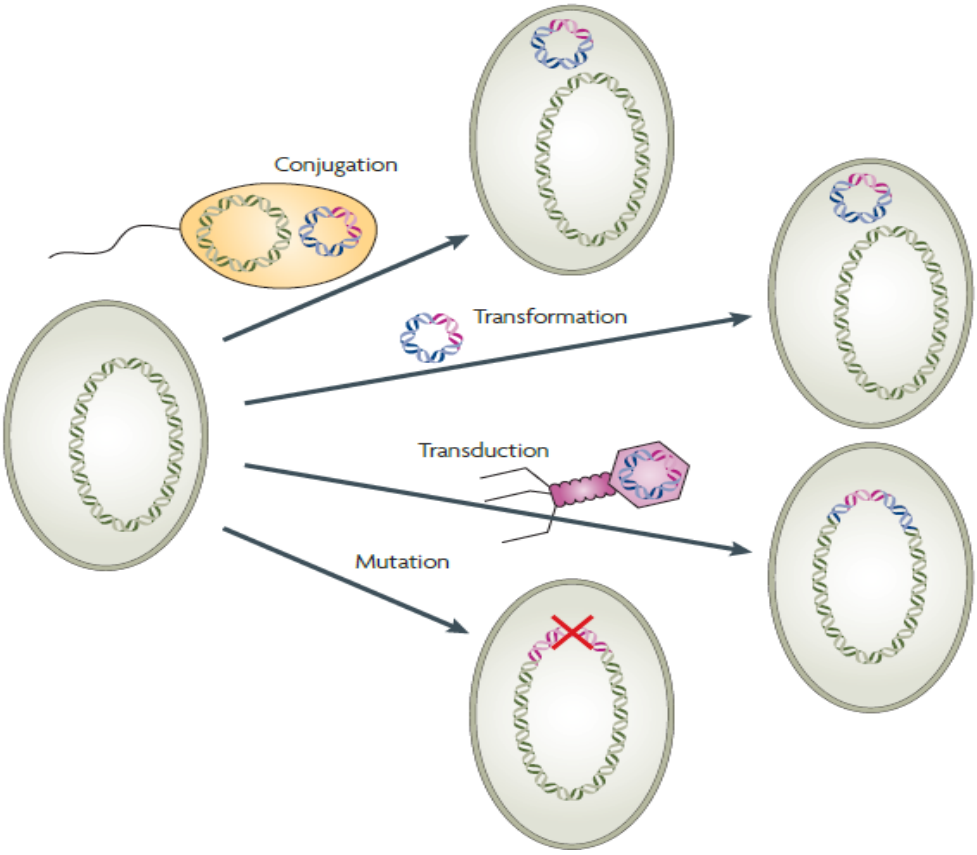

Figure 20. Mechanisms of resistance acquisition (Andersson and Hughes, 2010).

Natural transformation is the process where bacteria can assimilate exogenous DNA from the external environment (Avery *et al*., 1944). This process is observed in many Gram-negative and Gram-positive microorganisms. Conjugation is the process where mobile genetic elements (MGE) can be transferred between bacterial cells (reviewed by Toussaint and Chandler, 2012). Finally, transduction is the process where bacteriophages package host genes instead their own, and they transfer this exogenous genes into a new host (reviewed by Weinbauer and Rassoulzadegan, 2004). The HGT, mediated by any of the processes described above, is possible due to the Mobilome.

1.4.1. Mobilome

The Mobilome is defined as "all the mobile genetic elements (MGE) in a cell" (Siefert, 2009). In other words, the MGEs are the agents of HGT (Toussaint and Chandler, 2012). The Mobilome is composed of plasmids and transposable elements (Siefert, 2009).

The transposable elements group comprises transposons, insertion sequences (IS) and integrons, which, in turn, can be embedded into plasmids, allowing them to be mobilised between cells (Miriagou *et al*., 2006). They represent the most important genetic units involved in the mobilisation of genes, such as antibiotic-resistance genes, in bacteria. Transposons and IS (Figure 21) are genetic elements frequently associated with antibiotic-resistance genes (Miriagou *et al*., 2006).

1.4.1.1. Insertion sequences

An important part of the bacterial genome comprises IS elements (Figure 21). These MGEs are small (<2.5 kb), contain a transposase gene, an enzyme involved in their own mobility, and two inverted repeated sequences (IR) on the extremities (Mahillon and Chandler, 1998). These IR are short pieces of DNA (10-40 bp) whose functions are the binding of the transposase enzyme, cleavage and strand transfer reactions (reviewed by Mahillon and Chandler, 1998). They are composed by two functional domains. The domain A corresponds to the two or three terminal base pairs and is associated with cleavage and strand transfer reaction leading to the mobilisation of the IS whereas the domain B is involved in the transposase binding (Derbyshire *et al*., 1987). The IS play an important role in the process of antibiotic resistance. Many IS elements can regulate the expression of neighbouring genes. There are 25 IS elements described in *A. baumannii* to date (http://www-is.biotoul.fr, accessed on October 2013). An example of this effect is the case of IS*Aba1*, which is widely distributed in *A. baumannii*. This element has been found 7-bp upstream of the $bla_{\text{OXA-51-like}}$

gene, where this IS element provides a strong promoter for this carbapenemase gene, leading to its overexpression and resulting, finally, in a carbapenem-resistant strain (Turton *et al*., 2006a). IS*Aba1* has been associated with cephalosporin-resistance in *A. baumannii*, which has been detected upstream the $bla_{\text{ADC-like}}$ gene, whereupon its expression increased (Corvec *et al*., 2003). Although the main mechanism of controlling gene expression by IS elements is providing a strong promoter, these MGEs can also contribute to antibiotic resistance by a different mechanism; specifically, it has been demonstrated that IS*Aba10* in *A. baumannii* can affect the production of a porin called CarO, involved in antibiotic uptake, by disrupting the gene due to its insertion in the open-read frame (ORF) of *carO*, affecting the entrance of antibiotics from the outside (Lee *et al*., 2011).

1.4.1.2. Transposons

Transposons or "jumping genes" are a piece of DNA that encode functions related with their mobilisation and for an extra phenotype, such as, antibiotic resistance (Figure 21). There are two main classes of transposons according to their structure, class I or composite, and class II or complex transposons (Poirel *et al*., 2011).

Composite transposons are bracketed by two separate IS elements (Figure 21). The genetic unit spanning from one IS to another is mobilised entirely, transporting additional genes, such as antibiotic resistance. An example of this type of transposons corresponds to the Tn*5* transposon that is composed by two copies of IS*50* flanking three antibiotic resistance genes where one of the IS (right copy) copies encodes the transposase and an inhibitor of transposition, while the other copy (left copy) produces inactive proteins (reviewed by Reznikoff, 2003).

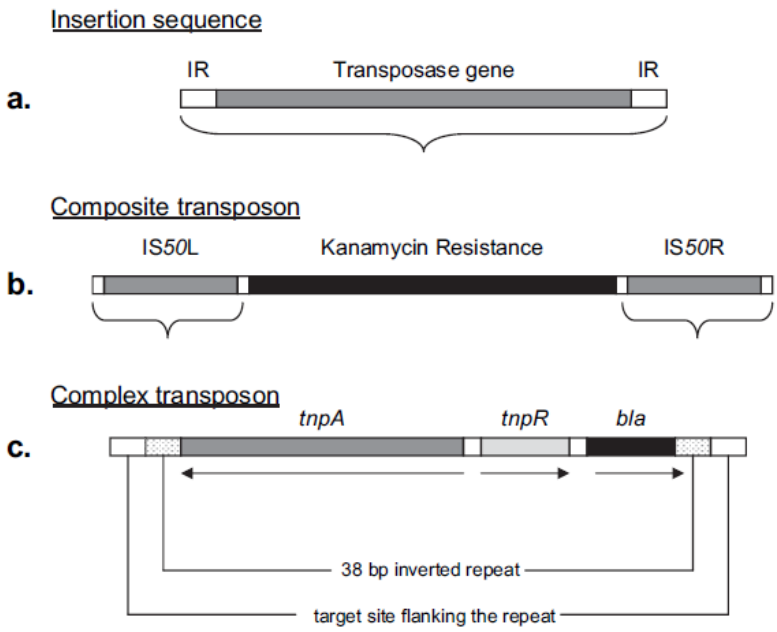

Figure 21. Schematic representation of transposons and insertion sequences (IS) (adapted from (Miriagou *et al*., 2006).

The complex transposons, such as Tn*2610*, are bracketed by a pair of short IRs on their ends (Figure 21). These IRs, unlike IS, lack transposition activity. The essential genes related with the functions of transposition are encoded in the central zone of these genetic units (Yamamoto, 1989). In addition to the genes related with mobilisation, the central non-repetitive zone of Tn*2610* carries genes of antibiotic resistance, specifically a β-lactamase gene together with streptomycin and sulfonamide resistance genes (Yamamoto *et al*., 1983).

1.4.1.3. Integrons

Integrons were described during the 1980s (Stokes and Hall, 1989). They are genetic structures capable of capturing and expressing genes that are embedded in mobile units called gene cassettes (Hall and Collis, 1995). An integron is not mobilisable itself, owing to the fact that it does not contain a transposase gene in its structure, but they are intimately associated with transposons, which allows them to be mobilised (Cambray *et al*., 2010). These genetic units constitute the major vectors in multidrug-resistance in Gram-negative pathogens (Partridge *et al*., 2009). All integrons comprise an integrase gene, *intI*, and are divided into different classes according to their nucleotide sequences (Hall and Collis, 1998). The integrase corresponds to a site-specific tyrosine recombinase that catalyses the capture/excision of the gene cassettes, such as antibiotic-resistance genes (Stokes and Hall, 1989) (Figure 22).

In order to fulfil their role of capturing and expression of gene cassettes, integrons comprise, in addition to *intI*, an *attI* recombination site where the cassette is inserted/excised by a site-specific recombination process between the *attI* and *attC* sequences and are transcribed from the promoter (Pc) provided by the integron unit (reviewed by Hall, 2012) (Figure 22).

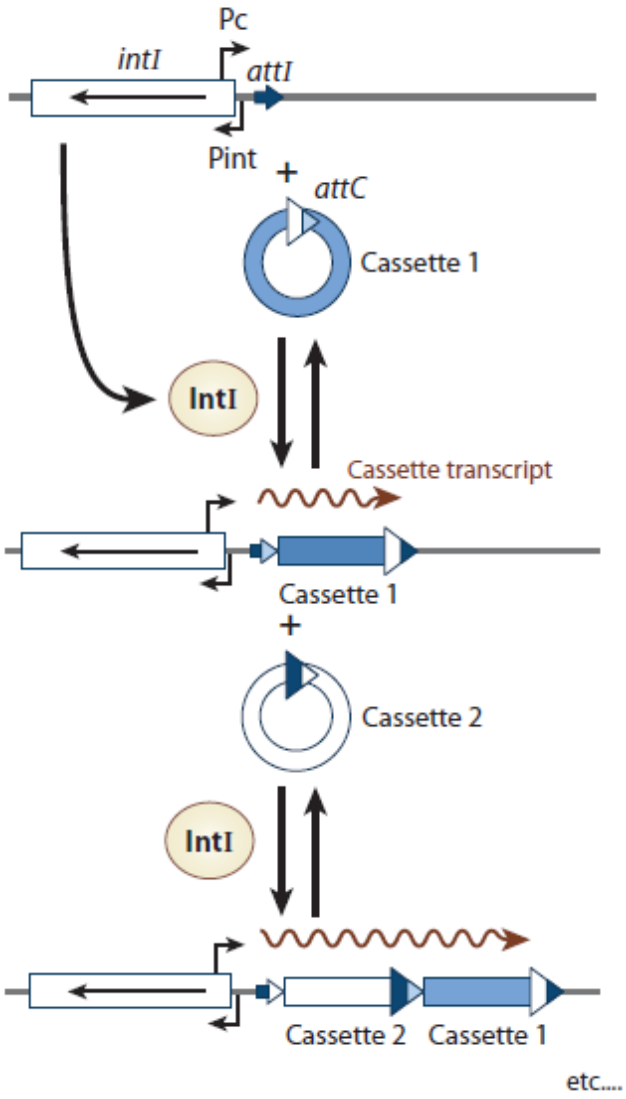

Figure 22. Integration/excision of gene cassettes by integrons (Cambray *et al*., 2010). Pc: strong promoter; *intI*: integrase-encoding gene; *attC*: recombination site (cassette); *attI*: recombination site (integron).

In addition to the classic integrons, another kind of integron-related genetic structures has been identified carrying antibiotic-resistance genes. These units, called IS*CR*-elements or complex-integrons, are characterised by the presence of a "common region" (CR) beyond the 3'-end of integron class 1 (Toleman *et al*., 2006). This CR region is classified as unusual

insertion sequences as they lack of terminal inverted repeated sequences and are mobilised by a rolling-circle transposition mechanism, permitting the transport of adjacent genes (Tavakoli *et al*., 2000). An example of this genetic unit is represented by IS*CR1* elements related with cephalosporins resistance in *A. baumannii*. In this case, the bla_{PER-7} gene, which confers resistance to ceftazidime, is located in a complex-integron that carries the classical structure of integrons class 1 but differs in the 3'-end by the presence of the CR element, called ORF513 (Opazo *et al*., 2012a) (Figure 23).

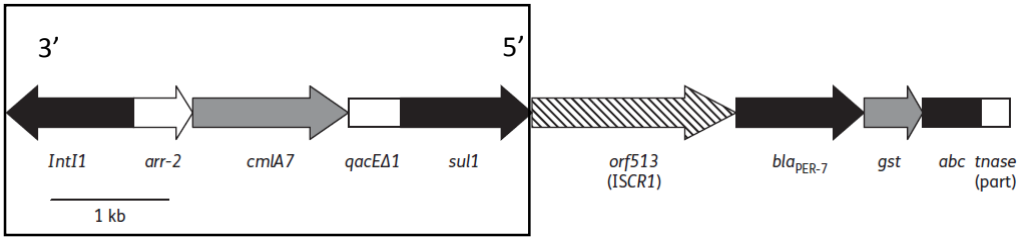

Figure 23. Schematic representation of an IS*CR1*-element. The classical structure of an integron class 1 is highlighted in the black box.

1.5. The genus *Acinetobacter*

The name *Acinetobacter*, from the Greek "akineto" which means "nonmotile", was initially proposed in 1954 in order to separate them from the motile genus *Achromobacter* (Brisou and Prevot, 1954). After a study published by Baumann *et al*. (Baumann *et al*., 1968) the genus was accepted by the committee of Taxonomy of *Moraxella* and Allied Bacteria (reviewed by Peleg *et al.*, 2008). Nowadays, the genus *Acinetobacter* includes Gram negative coccobacilli, with a DNA G+C content of 39 to 47%, nonmotile, catalase positive, strictly aerobic and oxidase negative (Bergogne-Bérézin and Towner, 1996). In the beginning, *Acinetobacter* was classified in the family *Neisseriaceae*, including one species, *Acinetobacter calcoaceticus.* Later, it was classified in the *Moraxellaceae*, which also includes *Moraxella, Psychrobacter* and related species (reviewed by Bergogne-Bérézin and Towner, 1996). This early classification was based on analysis of phenotypic characteristics which did not distinguish closely related species. Nevertheless, by the use of genotypic techniques, such as sequencing of the 16S rDNA, amplified fragment length polymorphism (AFLP) and sequencing of the *rpoB* gene, new species have been identified (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008). A comprehensive list of *Acinetobacter* species is represented in Table 2.

Most members of the genus *Acinetobacter* have been detected in human specimens (Table 2), but not all are considered as clinically important. In this sense, A. *baumannii*, *A. pittii* and *A. nosocomialis*, named the "*A. baumannii* complex", are responsible for causing opportunistic infections and outbreaks that involved *Acinetobacter* spp. mainly affecting immunocompromised patients (reviewed by Towner, 2009).

Table 2. Taxonomic classification of the genus *Acinetobacter* (Dijkshoorn *et al*., 2007; Nemec *et al*., 2009, 2010, 2011; Towner, 2009; http://www.bacterio.net/*acinetobacter*.html, last accessed June 4th, 2014).

Species Name	Genomic species	Source
Acinetobacter calcoaceticus	1	Humans (including clinical specimens) and soil
Acinetobacter baumannii	2	Humans (including clinical specimens)
Acinetobacter haemolyticus	4	Humans (including clinical specimens)
Acinetobacter junii	5	Humans (including clinical specimens)
Acinetobacter johnsonii	7	Humans (including clinical specimens)
Acinetobacter Iwoffii	8,9	Humans (including clinical specimens)
Acinetobacter radioresistens	12	Humans (including clinical specimens), soil and cotton
Acinetobacter ursingii		Humans (including clinical specimens)
Acinetobacter schindleri		Humans (including clinical specimens)
Acinetobacter parvus		Humans (including clinical specimens) and animals
Acinetobacter baylyi		Activated sludge and soil
Acinetobacter bouvetii		Activated sludge
Acinetobacter towneri		Activated sludge
Acinetobacter tandoii		Activated sludge
Acinetobacter tjernbergiae		Activated sludge
Acinetobacter gerneri		Activated sludge
Acinetobacter beijerinckii		Humans, soil and animals
Acinetobacter gyllenbergii		Humans (including clinical specimens)
Acinetobacter pittii	3	Humans (including clinical specimens), vegetables and soil
Acinetobacter nosocomialis	6	Humans (including clinical specimens)
	13TU	Humans (including clinical specimens)
Acinetobacter bereziniae	10	Humans (including clinical specimens), vegetables and soil
Acinetobacter guillouiae	11	Humans (including clinical specimens) and soil
	13BJ, 14TU	Humans (including clinical specimens)
	14BJ	Humans (including clinical specimens)
	15BJ	Humans (including clinical specimens)
	16	Humans (including clinical specimens) and vegetables
	17	Humans (including clinical specimens) and soil
	15TU	Humans (including clinical specimens) and animals
	"Between 1 and 3"	Humans (including clinical specimens)
	"close to 13"	Humans (including clinical specimens)

Table 2 (continued)

Species Name	Genomic species	Source
Acinetobacter boissieri		Plants
Acinetobacter brisouii		Activated sludge
Acinetobacter grimontii		Activated sludge
Acinetobacter harbinensis		Water
Acinetobacter indicus		Soil
Acinetobacter kooki		Soil
Acinetobacter nectaris		Plants (nectar)
Acinetobacter puyangensis		Trees (*Populus x euramericana*)
Acinetobacter qingfengensis		Trees (*Populus x euramericana*)
Acinetobacter rudis		Raw milk and raw waste water
Acinetobacter soli		Soil
Acinetobacter venetianus		Water

1.5.1. Typing techniques

Due to the difficulties to distinguish different *Acinetobacter* species, several techniques have been proposed for discriminating some or all members of this species; for example, four species (*A. calcoaceticus, A. baumannii*, *A. pittii* and *A. nosocomialis*) are very similar phenotypically, therefore they can only be distinguished by utilising molecular techniques (Evans *et al*., 2013). One proposed molecular method corresponds to the tRNA spacer fingerprinting, which is based on the amplification of the spacer regions of the tRNA (tDNA) clusters, which generates different electrophoretic patterns according to the specific sequences present on each species analysed (Ehrenstein *et al*., 1996; Evans *et al*., 2013).

Another method corresponds to the sequencing of the 16S rDNA, which is based on the amplification and sequencing of the bacterial 16S rDNA (Ibrahim *et al*., 1997). In addition, a further method based on the 16S rDNA sequence is the amplified 16S ribosomal DNA

(rDNA) restriction analysis (ARDRA), in which, unlike the method described above, the amplification products are digested by different restriction enzymes (normally 5 enzymes), generating specific patterns for each species (Dijkshoorn *et al*., 2007). Another method based on the bacterial rDNA profile is the restriction analysis of the 16S-23S rDNA region, which examines the polymorphism within rRNA (rDNA) intergenic spacer regions present on the different *Acinetobacter* species (Dolzani *et al*., 1995). Amplified fragment length polymorphism (AFLP) analysis, in which the whole bacterial DNA is digested by restriction enzymes and then the fragments are PCR-amplified, generating specific patterns for each species (Dijkshoorn *et al*., 2007). This technique is also utilised to perform population structure studies within *A. baumannii*.

Two methods based on the sequence of specific genes are the sequencing *gyrB* and *rpoB* genes. Both genes are considered as "housekeeping genes" and they represent appropriate candidates for species identification (Evans *et al*., 2013; Gundi *et al*., 2009; Peleg *et al*., 2008). The "housekeeping genes" are constitutive genes that are necessary to maintain the basic cellular functions and are under purifying selection and slow evolution, and the changes on these genes are almost neutral (Enright and Spratt, 1999). The use of these methods has increased latterly, as they are rapid and reliable alternatives for identification.

Another method proposed, is represented by the multilocus PCR and mass spectrometry (PCR/ESI-MS), where six "housekeeping genes" are amplified by PCR and their sequences are obtained by electrospray ionisation mass spectrometry (Ecker *et al*., 2006). Even though this method represents a rapid and reliable alternative, it needs special equipment and trained personal, which makes it more expensive than other methods.

Despite the existence of several methods proposed, as mentioned previously, there is no universal consensus as to which should be used. The large number of methods that are used in different laboratories makes species-identification a problematic matter, as methods change in their specificity and sensitivity, making inter-laboratory comparisons unreliable (Evans *et al*., 2013).

1.5.2. *Acinetobacter baumannii*

In epidemiological surveys, *A. baumannii* have been identified as the most frequent cause of infections among *Acinetobacter* species (reviewed by Evans *et al*., 2013). Although it has been considered that members of this genus are ubiquitous (Table 2), some being isolated from the environment and some form part of the normal human microbiota, *A. baumannii* has been recovered almost exclusively from nosocomial environment (reviewed by Towner, 2009).

A. baumannii is a microorganism with a low pathogenicity, in which the colonisation rate is higher than the infection (Dijkshoorn *et al*., 2007). Nevertheless, the crude mortality rates, associated with infections caused by this microorganism, are higher (between 8% and 43%) compared with other *Acinetobacter* species (between 7% and 18%) (Choi *et al*., 2006; Falagas *et al*., 2006; Seifert *et al*., 1994). Various factors contribute in the colonisation process, such as its adherence to the host cells, resistance to inhibitory agents and biofilm formation (reviewed by Dijkshoorn *et al*., 2007). Then, when the infection starts, it can be severe. Once the pathogen has colonised the patient, various factors play important roles in the infection process. Specifically, the LPS is a potent proinflammatory molecule, which stimulates an inflammatory signaling reaction through human Toll-like receptors (Erridge *et al*., 2007) and the outer membrane protein A (AbOmpA) possesses a cytotoxic activity (Choi

et al., 2008). Additionally, iron-capturing mechanisms and resistance to human serum help *A. baumannii* to survive a long time in the blood during bloodstream infections (Dorsey *et al*., 2003; Jankowski *et al*., 1992).

1.5.2.1. Population structure of *A. baumannii*

Diverse typing techniques, as AFLP, have shown genotypic diversity within *A. baumannii* (Diancourt *et al*., 2010). In this sense, multilocus sequence typing (MLST) is considered as the "gold standard" method to investigate the population structure and global spread of microbial pathogens. In this method, seven "housekeeping genes" are amplified and sequenced, generating diverse Sequence Types (STs) which are compared with those STs contained in an international database (Bartual *et al*., 2005; Diancourt *et al*., 2010). MLST studies have revealed the main structure of MDR-*A. baumannii* population, where 3 different International clones (ICs), also called "worldwide" (WW) lineages, are predominant (Diancourt *et al*., 2010; Higgins *et al*., 2010). Indeed, the ICs-I, -II and -III are highly prevalent worldwide (Diancourt *et al*., 2010). However, according to Zarrilli *et al.*, the population structure of *A. baumannii*, is changing, showing that it comprises at least nine different clonal lineages, which can disseminate in single institutions and/or worldwide (Zarrilli *et al*., 2013). Specifically, the IC-I and –II prevail internationally, while the IC-III other clonal lineages, such as the clonal complex 10 and clonal complex 15, are more prevalent in European hospitals (Zarrilli *et al*., 2013). Interestingly, each IC is related to a specific $bla_{\text{OXA-51-like}}$ enzyme; for example the IC-I is related to $bla_{\text{OXA-69}}$, whereas the IC-II is associated with $bla_{\text{OXA-66}}$ and the $bla_{\text{OXA-71}}$ is present in isolates belonging to the IC-III (Zander *et al*., 2012), making it possible to relate the presence of specific $bla_{\text{OXA-51-like}}$ genes with some ICs (Pournaras *et al*., 2014), although this is still under discussion. It should be noted that the current population studies of *A. baumannii* have been performed by two different MLST schemes (Zarrilli *et al*., 2013), which strongly indicates that is essential to

standardise the methodology, in order to generate a more complete understanding of the dissemination of MDR ICs.

1.5.2.2. Clinical infections caused by *A. baumannii*

A. baumannii is classified as a nosocomial pathogen involved in infections that mainly affect critically ill patients (Dijkshoorn *et al*., 2007). The fact that the colonisation is more common than infection, reflects that this microorganism possesses low pathogenesis (Dijkshoorn *et al*., 2007). However, some serious clinical infections are associated with this pathogen, in particular hospital-acquired pneumonia which is the most common infection caused by *A. baumannii* (McConnell *et al*., 2013). The ventilator-associated pneumonia (VAP) occurs mostly in critical patients who are receiving mechanical ventilation; factors such as longer periods of hospitalisation, longer time under mechanical ventilation, previous use of antibiotics (Howard *et al*., 2012), health care professionals with colonised hands and poor hygiene (Peleg *et al*., 2008) and contaminated care equipment all increase the risk of developing this infection (Luna and Aruj, 2007). Importantly, the crude mortality associated with VAP caused by this pathogen has been reported to vary between 40% and 70% (Garnacho *et al*., 2003). Besides the VAP mentioned above, cases of community-acquired pneumonia associated with *A. baumannii* have been reported in Asia and Australia (Anstey *et al*., 2002). The crude mortality associated with this type of pneumonia ranges between 40% and 60% (Leung *et al*., 2006), often being related with underlying host factors, such as alcohol consumption or chronic obstructive pulmonary disease (McConnell *et al*., 2013).

In addition to pneumonia, *A. baumannii* is associated with bloodstream infections (BSI) in the intensive care units. The common sources of BSI are lower respiratory tract infections and intravascular devices, although other primary infections, such as wound infections and

urinary tract infections have also been associated with BSI *(McConnell et al*., 2013). The crude mortality associated to BSI ranges between 28% and 43% (McConnell *et al*., 2013; Wisplinghoff *et al*., 2004).

A. baumannii is also an important cause of burn and soft tissue infections, which are complicated to treat because the strains causing these infections are usually MDR and some antibiotics have a poor penetration to the infected sites (McConnell *et al*., 2013). Interestingly, *A. baumannii* has been associated with wound infections in military personnel. Specifically, in a study carried out at the US Army Institute of Surgical Research Burn Center, *A. baumannii* represented 22% of the microorganisms recovered from burn injuries, where 56% of the isolates were MDR (Keen *et al.*, 2010); however, these infections are uncommon outside the military environment (Gaynes and Edwards, 2005). These infections are complicated to treat, as they could produce cellulitis and necrosis, requiring surgical removal of the infected tissue in addition to antibiotic therapy (Sebeny *et al*., 2008).

Meningitis can also be caused by *A. baumannii*. This infection is commonly associated with patients recovering from neurosurgical procedures (McConnell *et al*., 2013); though, due to the limited number of studies, the rates of crude mortality are still unclear. It has been reported as high as 70% in a study published in 2007 (Metan *et al*., 2007). Finally, *A. baumannii* has been rarely identified causing endocarditis, where it was associated with prosthetic valves (Olut and Erkek, 2005) and intravascular catheters (Bhagan-Bruno *et al*., 2010).

1.5.2.3. Genomic islands (GIs)

In general terms, genomic islands (GIs) are defined as clusters of genes for which there is evidence of horizontal origins (Langille *et al*., 2010). There are different subtypes of GIs, classified according to the functions encoded by the genes they contain; for example "secretion islands", antimicrobial "resistance islands" and "metabolic islands" (Langille *et al*., 2010). Consequently, the term "genomic island" has since been utilised as a general concept that referred to any cluster of genes, normally 10-200 kb in length that has been obtained through horizontal transfer (Hacker and Kaper, 2000). The basic structure of the GIs (Figure 24) includes the presence of direct repeat sequences flanking them, an internal region containing diverse genes encoding diverse features, functional and fragmented IS elements and other mobility-related elements, as well as a functional integrase gene, which is the responsible in the insertion or deletion of DNA in the central area of the GIs (Dobrindt *et al*., 2004). GIs are often integrated at tRNA genes in the bacterial chromosome (Figure 24) and often harbour functional or cryptic genes related to plasmid conjugation systems or phages involved in the mobilisation of the complete structure (Juhas *et al*., 2009). The life cycle of the GIs consists of the acquisition of the GI by horizontal transferring, integration of this element into the host chromosome by specific-recombination catalysed by an integrase, development of the GI by rearrangements, gene acquisition or loss of other mobile genetic elements, excision of the GI and finally transferring to another recipient cell (Juhas *et al*., 2009).

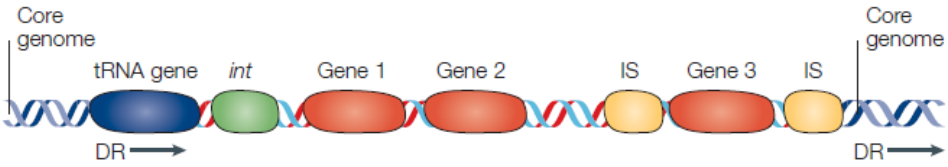

Figure 24. General characteristics of GIs. DR: direct repeat; IS: Insertion sequence (Dobrindt *et al*., 2004).

Even though the reason why GIs are often inserted into tRNA genes is still unclear, it is known that the insertion of these elements is catalysed by site-specific phage-like recombinases (integrases), which are usually present in the GI itself. Several of these integrases are related to the lambda, P4 or XerD families (Juhas *et al*., 2009; van der Meer *et al*., 2001), which may suggest the importance of the role of phages in the origin of GIs. An important characteristic of the GIs is that they have a lower G+C content in comparison with the core genome, reaffirming that the GIs presumably are horizontally acquired (Juhas *et al*., 2009).

The first GI in *A. baumannii* was characterised in 2006, in France, in an MDR-strain isolated. The strain, named AYE, was resistant to β-lactams (excluding imipenem, piperacillin-tazobactam and ticarcillin-clavulanate), aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline and rifampin (Fournier *et al*., 2006). This GI was specifically characterised as a resistance island (RI) as it harboured several genes of antibiotic and metal resistance (Fournier *et al*., 2006). This RI, termed AbaR1, is the largest GI described to date, as it has a length of 86-kb, encoding 88 predicted open-read frames (ORFs) of which 45 were associated with resistance to antibiotics or biocides (Evans *et al.*, 2013; Fournier *et al*., 2006; Krizova and Nemec, 2010). Interestingly, unlike most of the GIs, the AbaR1 island

was inserted into the *comM* gene, which corresponds to an ATPase gene (Krizova and Nemec, 2010).

Fournier *et al*. also analysed a susceptible strain, named SDF, which also had a disruption in the *comM* gene. In this case, a GI of 19-kb in length was detected, which contained 25 ORFs, none of which was associated with antimicrobial resistance (Fournier *et al.*, 2006). Remarkably, the resistance genes identified in AbaR1 are likely to have originated in different species, according to the amino acid sequences of the proteins encoded by them, 44% are likely originated from *Pseudomonas* spp., 34% from *Salmonella* spp., 17% from *Escherichia* spp. and 4% from different microorganisms (Fournier *et al*., 2006). This demonstrates that this RI is the result of successive acquisition of determinants of resistance from different hosts (Fournier *et al*., 2006; Schmidt and Hensel, 2004). Nowadays, several distinct RIs have been identified in *A. baumannii* strains, such as AbaR2, which was detected in an isolate collected in 2005 in Rome that harboured 17 genes, 7 of which are related to antimicrobial resistance (Iacono *et al*., 2008). Another RI was detected in an *A. baumannii* strain isolated in the USA, which contained a RI of 63-kb in length that harboured 8 resistance genes (Adams *et al*., 2008). Interestingly, a strain collected in 1977 was found to contain a 63-kb RI highly similar to AbaR3, suggesting that these structures have been present in *A. baumannii* for a long time (Krizova and Nemec, 2010), reflecting the ability of *A. baumannii* to capture and accumulate resistance genes, making it a difficult pathogen to treat.

1.5.1.1 Multidrug-resistant *Acinetobacter baumannii*

The antibiotics available to treat infections caused by *A. baumannii* include broad-spectrum penicillins, broad-spectrum cephalosporins, monobactams, fluoroquinolones, aminoglycosides, polymyxins, carbapenems, sulbactam and tigecycline (reviewed by Towner, 2009). Nevertheless, the appearance of multidrug-resistant strains has increased during the last decade. In the case of *A. baumannii*, a multidrug-resistant (MDR) isolate is resistant to more than two of the following drugs: cephalosporins (ceftazidime or cefepime), carbapenems (imipenem or meropenem), ampicillin-sulbactam, fluoroquinolones (ciprofloxacin or levofloxacin) and -aminoglycosides (gentamicin, tobramycin or amikacin) (Peleg *et al.*, 2008). Importantly, the increase of MDR isolates of *A. baumannii* worldwide is limiting the therapeutic options for treating infections caused by this MDR pathogen (Durante-Mangoni and Zarrilli, 2011).

The MDR phenotype of *A. baumannii* is due to its remarkable ability to upregulate innate resistance mechanisms and to acquire exogenous resistance genes that play an important role in the development of this phenotype (Howard *et al*., 2012).

There are several mechanisms of antibiotic resistance identified in *A. baumannii* that provide it with the ability to survive under the pressure from a wide range of antibiotics (Table 3), which, as mentioned before, could be associated with unique genetic structures, such as GIs.

Table 3. Mechanisms of antibiotic resistance in *A. baumannii* (Bonnin *et al*., 2011a; Doi *et al*., 2004; Durante-Mangoni and Zarrilli, 2011; Evans *et al*., 2013; Higgins *et al*., 2013; Kaase *et al*., 2011; Peleg *et al*., 2008; Poirel *et al*., 2011; Robledo *et al*., 2010; Tian *et al*., 2011).

Antimicrobial drugs	Mechanisms of antibiotic resistance and classes of proteins involved	Examples of specific enzymes, genes and targets involved
Cephalosporins	**β-lactam hydrolysis**	
	Class C β-lactamase	ADC-1, -2, -3, -4, -5, -6, -7.
	ESACs	ADC-33,-56.
	Class A ESBL	VEB-1,-1a; PER-1,-2,-7; TEM-92,-116; SHV-5,-12; CTX-M-2,-3,-43; GES-11;-12; RTG-4.
	Class D β-lactamase	OXA-51-like enzymes.
Carbapenems	**β-lactam hydrolysis**	
	Class A β-lactamase	KPC-2,-3,-4,-10; GES-4,-5,-6,-14.
	Class B MBLs	IMP-1,-2,-4,-5,-6,-11; VIM-1,-2; SIM-1; NDM-1,-2.
	CHDLs	OXA-23, -40, -58, -143 and -235 clusters.
	OXA-51-like class D β-lactamase	Confers resistance if there is a IS upstream of the gene.
	Changes in OMPs	
	CarO	*carO* gene regulated by IS elements.
	OprD-like OMP and 33-36 kDa OMP	Other OMPs involved in carbapenem-resistance.
Aminoglycosides	Aminoglycosides-modifying enzymes (AMEs)	AacC1/2; AadA; AadB; Ant1; AphA1; AphA6; AphA1B; Aac(6')-Iad.
	16s rDNA methyltransferase	ArmA
Quinolones	**Target modification**	
	Gyrase subunit	Mutation of the *gyrA* gene
	Topoisomerase IV subunit	Mutation of the *parC* gene
Rifampicin	**Drug modification**	Arr-2
	Target modification	Mutation of the *rpoB* gene
Cotrimoxazole	**Replacement of target**	
	Dihydropteroate synthase (DHPS)	Presence of *sul1* and/or *sul2* gene(s)
	Dihydrofolate reductase (DHFR)	Mutation of the *folA* gene
		Presence of *dfr* genes.

Table 3. (continued)

Antimicrobial drugs	Mechanisms of antibiotic resistance and classes of proteins involved	Examples of specific enzymes, genes and targets involved
Broad (aminoglycosides, quinolones, tetracyclines, glycylcyclines)	**Efflux**	
	RND-family	AdeABC; AdeFGH; AdeIJK
	MATE-family	AdeM
	MFS-family	TetA; TetB
Polymyxins	**Outer membrane modification**	Mutation in genes of lipid A synthesis and PmrAB two components system.

The mechanisms involved in the resistance to aminoglycosides include the presence of aminoglycosides-modifying enzymes (AMEs) which have been detected in integron class 1 elements, including acetyltransferases, nucleotidyltransferases and phosphotransferases (reviewed by Peleg *et al.*, 2008). Even though the presence of AMEs is the most prevalent mechanism of aminoglycosides in this pathogen, the methylation of the 16s rRNA, mediated by the ArmA enzyme, represents a new mechanism conferring a high-level resistance to all aminoglycosides currently available (Durante-Mangoni and Zarrilli, 2011; Yu *et al*., 2007).

Resistance to tetracyclines and their derivatives (i.e. glycylcyclines) is mediated mainly by efflux pumps (Fluit *et al*., 2005). Two specific efflux pumps, TetA and TetB (Table 3), have been described in *A. baumannii* (Guardabassi *et al*., 2000; Ribera *et al*., 2003). The *tet(A)* gene has been detected in a transposon similar to *Tn1721*, which allows it to mobilise between different isolates (Ribera *et al*., 2003). Neither of them, TetA and TetB, affects tigecycline, which is expelled by the overexpression of the efflux pump AdeABC (Ruzin *et al*., 2007). In addition to AdeABC, AdeFGH and AdeIJK can expel a broad spectrum of antibiotics, including tigecycline (reviewed by Durante-Mangoni and Zarrilli, 2011).

Quinolone-resistance is mediated by modifications of the DNA gyrase or topoisomerase IV by mutations in *gyrA* and *parC* genes that interfere with the binding between the antimicrobial drug and the target (reviewed by Peleg *et al*., 2008). It has been demonstrated that a single mutation in the *gyrA* gene at codon Ser-83 increases the MIC of ciprofloxacin from 2 mg/L to >32 mg/L (Spence and Towner, 2003). Additionally, an extra mutation in the codon Ser-80 of the *parC* gene leads to moxifloxacin resistance (Spence and Towner, 2003). Also, quinolones can be extruded by activity of broad spectrum RND-efflux pumps (Durante-Mangoni and Zarrilli, 2011; Higgins *et al*., 2004) and by the MATE pump AbeM (Su *et al*., 2005).

Polymixin resistance is a new mechanism of resistance identified in *A. baumannii* as this drug have been reintroduced during the last few years for treating serious infections caused by MDR isolates. The mechanism of resistance includes the complete loss of the lipid A, which is a component of the LPS. This loss is a consequence of mutations within one the genes involved in the biosynthesis of the lipid A: *lpxA*, *lpxC* and *lpxD* (Moffatt *et al*., 2010). Moreover, polymixin resistance can be mediated by mutations in the PmrAB system. This is a system involved in sensing environmental pH, Fe^{3+} and Mg^{2+} levels, leading to altered the expression of genes involved in the biosynthesis of the lipid A (Adams *et al.*, 2009). In colistin resistant *A. baumannii* strains, the expression of *pmrA* is increased, indicating that the mutations in this gene leads to a constitutive expression of PmrA, with the consequence that the strains become resistant to this drug (Adams *et al*., 2009).

In the case of rifampicin, the major mechanism of resistance corresponds to mutations in the *rpoB* gene that encodes the β-subunit of RNA polymerase (McCammon *et al*., 2005; Telenti *et al*., 1993). These mutations occur in and outside the rifampin resistance determining regions

(RRDR) of the *rpoB* gene, interrupting the binding of the drug to the target site (Heep *et al*., 2001; Tribuddharat and Fennewald, 1999). However, this mechanism has been identified mainly in *M. tuberculosis*, whereas the molecular basis of rifampicin resistance in *A. baumannii* and other Gram negative pathogens is typically due to the presence of the *arr-2* gene, which is usually part of integrons class 1 elements (Thapa *et al*., 2009; Tribuddharat and Fennewald, 1999). This additional mechanism of rifampicin resistance includes the presence of a rifampicin ADP-ribosylating transferase, named Arr-2, which inactivates rifampicin by ribosylation (Poirel *et al*., 2011). The fact that this gene is usually associated with integron class 1 structures, facilitates its spread among different isolates (Houang *et al*., 2003).

The resistance to trimethoprim is due to mutations of the *folA* gene, which produces a dihydrofolate reductase (DHFR) that is not affected by trimethoprim, conferring resistance to this antibiotic (Mak *et al*., 2009). Additionally, the resistance to trimethoprim can be due to the presence of *dfr* genes, which can be present either on the chromosome or plasmids. These genes encode for trimethoprim-resistant DHFRs, obstructing the activity of this antibiotic (Sköld, 2000). Furthermore, integrons class 1 are very common among MDR strains of *A. baumannii* (Peleg *et al*., 2008). The 3'-conserved region of these genetic elements contains the *qacE* and the *sul1* genes, which confer resistance to antiseptics and sulphonamides, respectively (Walsh *et al*., 2005). The *sul1* gene encodes a dihydropteroate synthase (DHPS) that is not affected by sulphonamides (Antunes *et al*., 2005). Besides the *sul1* gene, the *sul2* gene, which encodes for a variant of the DHPS, has been identified mediating the resistance to sulphonamides. This gene is mainly located on small plasmids, thus facilitating its spread (Sköld, 2001).

In the case of β-lactams, the most prevalent mechanism of resistance is the enzymatic hydrolysis by β-lactamases. Intrinsically, all *A. baumannii* harbour an AmpC-like enzyme, called ADC (*Acinetobacter*-derived cephalosporinase) (Hujer *et al*., 2005). These enzymes are capable to hydrolyse cephalosporins, including extended-spectrum cephalosporins such as ceftazidime, when there is an IS element upstream of them, that increases their expression (Corvec *et al*., 2003). This represents the most common mechanism of cephalosporins resistance in *A. baumannii*. Nonetheless, the ADC enzymes do not possess activity against carbapenems and cefepime (Hujer *et al*., 2005). However, there are new ADC variants with activity against all cephalosporins, including cefepime. They include ADC-33 and ADC-56 and have been designated as ESAC (extended-spectrum AmpCs) (Rodríguez-Martínez *et al*., 2010; Tian *et al*., 2011). Unlike other ADC members, ESACs do not need the presence of an IS element upstream to promote the degradation of cephalosporins but they have two aminoacidic changes in the Ω-loop, increasing their spectrum of activity (Rodríguez-Martínez *et al.*, 2010; Tian *et al*., 2011). Additional mechanisms of cephalosporin resistance are less prevalent in *A. baumannii*. These include the presence of class A ESBLs with activity against extended-spectrum cephalosporins, such as members of PER (Opazo *et al*., 2012a), GES (Delbrück *et al*., 2012), SHV, VEB, TEM and CTX-M families (Table 3) (reviewed by Durante-Mangoni and Zarrilli, 2011). Most of these ESBLs have been identified embedded in diverse types of mobile genetic elements. TEM-like ESBLs have been detected in the transposons *Tn1*, *Tn2* and *Tn3* (reviewed by Bonnin *et al.,* 2011b). $bla_{\text{SHV-like}}$ genes have been identified bracketed by two copies of IS*26*, which may play a role in the mobilisation of these genes (Ford and Avison, 2004). Several types of genetic elements are related with mobilisation of CTX-M-like ESBLs, such as IS*Ecp1* and IS*CR1* and also phage-related elements (Poirel *et al.*, 2003; Poirel *et al*., 2008). In the case of VEB and GES-type ESBLs, their genes have formed part of integrons class 1 (reviewed by Poirel *et al.*, 2008), while PER-like enzymes have been identified as part of a composite transposon called *Tn1213* (Poirel *et al*., 2005a) and also associated with IS*CR1* (Opazo *et al*., 2012a).

1.5.1.2 Carbapenem-resistance in *Acinetobacter baumannii*

Carbapenems, such as imipenem and meropenem, are a potent group of β-lactams which represent one of the last options for treating infections caused by MDR *A. baumannii* due to their good activity and low toxicity (Evans *et al*., 2013; Towner, 2009). They have the broadest spectrum of activity within the β-lactam drugs (Nicolau, 2008). Nevertheless, as in other species, detection of carbapenem-resistant isolates has increased worldwide during the last decade. Even though carbapenem-resistance is mediated by various mechanisms and is the result of synergy between these mechanisms (Table 3), the most important is represented by enzymatic hydrolysis by carbapenemases (reviewed by Durante-Mangoni and Zarrilli, 2011). The β-lactamases with carbapenemase activity include class D OXA-type β-lactamases, metallo β-lactamases (MBLs) and class A β-lactamases (Table 3).

The carbapenem-hydrolysing class D oxacillinases (CHDLs) are the most prevalent carbapenem-hydrolysing enzymes in *A. baumannii* and are represented by the OXA-type β-lactamases. The CHDLs are divided in six different clusters, according to their nucleotide sequences (Evans *et al*., 2013; Higgins *et al*., 2013). These clusters are chromosomally encoded OXA-51-like and the acquired OXA-23-like, OXA-58-like, OXA-40-like (reviewed by Evans *et al*., 2013), OXA-143-like (Higgins *et al*., 2009) and OXA-235 (Higgins *et al*., 2013).

The OXA-51-like enzymes are mainly chromosomally encoded enzymes and are considered as intrinsic to *A. baumannii* (Turton *et al*., 2006a). The first member of this group was identified in Argentina in 2005 in carbapenem-resistant strains isolated from 1996 (Brown *et al*., 2005). There are nearly 100 variants of OXA-51-like enzymes identified, representing the largest CHDLs group (Evans *et al*, in press). Even though most of the members of this

cluster have been identified in the chromosome of *A. baumannii*, some studies have detected OXA-51-like β-lactamase genes in plasmids (Pournaras *et al*., 2006; Vahaboglu *et al*., 2006). The role of the OXA-51-like enzymes in the resistance to carbapenems depends in their over-expression as a consequence of the presence of promoters in IS elements, such as IS*Aba1*, upstream the *bla*$_{\text{OXA-51-like}}$ gene (Turton *et al*., 2006a).

The very first OXA-type enzyme with carbapenemase activity was detected in 1993 in Edinburgh, in a strain collected in 1985. This enzyme was originally designated as ARI-1 and later re-named as OXA-23 (Donald *et al*., 2000; Paton *et al*., 1993). There are four members of this cluster identified to date in *A. baumannii*, which have been detected both in plasmid and chromosome, while OXA-23-like enzymes have been detected in various members of *Acinetobacter* genus and also in *Proteus mirabilis* and *Klebsiella pneumoniae* (Bogaerts *et al*., 2006; Bonnet *et al*., 2002; Evans *et al*., 2013). Another OXA-type carbapenemase cluster is represented by the OXA-40-like enzymes. OXA-40 enzyme was detected in a Spanish hospital in 1997 (Bou *et al*., 2000). There are six variants identified to date, from which four have been detected in *A. baumannii* (reviewed by Evans *et al*., 2013). The OXA-58-like cluster is represented by four variants identified in *A. baumannii*. The first member, OXA-58, was identified in a strain collected in France in 2003 (Poirel *et al*., 2005). Lately, two new OXA-type enzymes clusters have been characterised. One is represented by the OXA-143-like cluster, with OXA-143 itself and OXA-182 as unique members. OXA-143 was identified in 2009 in three isolates from Brazil, in a plasmid of ca. 30 kb (Higgins *et al*., 2009) while OXA-182 was identified in twelve isolates from Korea (Kim *et al*., 2010). Another new OXA-type carbapenemase cluster is represented by OXA-235-like enzymes, which includes the OXA-235, OXA-236 and OXA-237 enzymes. This new CHDLs were detected in plasmid from strains collected in the United States and Mexico between 2005 and 2009 and were flanked by two copies of IS*Aba1* both upstream and downstream (Higgins *et*

al., 2013). The prevalent CHDLs predominantly have specific geographic distributions, whereas OXA-51-like enzymes are detected worldwide, OXA-23-like carbapenemases have been detected in the UK, France, Brazil, Iraq, Greece, Singapore, Italy, China (Opazo *et al*., 2012b; Zarrilli *et al*., 2013). In addition, isolates of *A. baumannii* carrying variants of the OXA-40-like cluster have been frequently detected in Spain, Belgium, South Asia, Croatia, Brazil and Italy (reviewed by Zarrilli *et al*., 2013). Finally, members of the OXA-58-like cluster are often identified in plasmids and have been detected frequently among isolates collected worldwide (Pournaras *et al*., 2006).

The class B carbapenemases are metallo β-lactamases (MBLs) which are able to hydrolyse all β-lactams available, excepting aztreonam, and are not inhibited by β-lactamase inhibitors (reviewed by Walsh *et al*., 2005). Despite their higher activity in comparison with CHDLs, their prevalence is lower in *A. baumannii* (reviewed by Peleg *et al*., 2008). Four groups of MBLs have been identified in carbapenem-resistant *A. baumannii*: VIM, IMP, SIM (reviewed by Peleg *et al*., 2008) and latterly NDM-like MBLs (Espinal *et al*., 2013; Kaase *et al*., 2011; Nakazawa *et al*., 2013) have been detected in this microorganism. Interestingly, *A. baumannii* has an important role in the origin and spread of NDM-like MBL. Even though the first description of $bla_{\text{NDM-1}}$ was made in 2008 in *Enterobacteriaceae* isolates analysed in Sweden from a patient hospitalised in India; these enzymes have been also detected in *A. baumannii*, in particular $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-2}}$ (Espinal *et al*., 2013; Rafei *et al*., 2014). The sequence of the $bla_{\text{NDM-1}}$ gene suggests that it was originated in *A. baumannii* by the fusion of a pre-existing MBL gene with the AMEs gene *aph6*, resulting in a chimeric β-lactamase (Toleman *et al*., 2012).

It is suggested that this fusion occurred in this microorganism due to the fact that the *aph6* gene is strongly related to *A. baumannii* and was firstly associated to this microorganism (Toleman *et al*., 2012). Secondly, the IS*Aba125* element, which is originally from *A. baumannii*, is frequently associated upstream of this MBL gene. Thirdly, the first European isolate of *A. baumannii* harbouring the $bla_{\text{NDM-1}}$ gene, contained both $bla_{\text{NDM-1}}$ and IS*Aba125* transposon, with *aph6* (Toleman *et al*., 2012). All these findings suggest that the first $bla_{\text{NDM-like}}$ gene was generated in *A. baumannii* and their spread occurred from this microorganism.

MBLs are commonly associated with integrons, representing a platform of dissemination of these determinants of resistance (Houang *et al*., 2003; Lee *et al*., 2012; Mishra *et al*., 2013; Tsakris *et al*., 2006). Like CHDLs, MBLs have a specific geographical distribution, where isolates carrying enzymes of the IMP family have been identified in Italy, Hong Kong, Singapore, Portugal, Brazil and Japan (reviewed by Zarrilli *et al*., 2013). In addition, members of the VIM-like family have been detected in isolates from Europe and the Far East (Tsakris *et al*., 2006; Zarrilli *et al*., 2013) whereas the SIM-1 MBL has been identified in isolates from South Korea (Lee *et al*., 2005). Finally, members of the new group of MBLs, NDM-like enzymes, have been detected in India, Germany, Algeria, Belgium, Bangladesh, Czech Republic, China, Egypt, Israel and the United Arab Emirates (reviewed by Zarrilli *et al*., 2013).

In addition to MBLs and CHDLs, another group of carbapenemases have been detected in *A. baumannii*. These enzymes are rarely detected in this pathogen and are represented by class A β-lactamases (Table 3), specifically by GES and KPC enzymes (Bonnin *et al*., 2013; Robledo *et al*., 2010). Members of the KPC group have been detected in Puerto Rico

(Robledo *et al*., 2010) whereas GES-type carbapenemases have been identified in France and Belgium (Bonnin, *et al*., 2011b).

Besides the presence of carbapenemases, there are other non-enzymatic mechanisms which play a role in the carbapenem resistance in *A. baumannii*. This mechanisms include modifications in the outer membrane proteins involved in the uptake of antibiotics from the external environment (Peleg *et al.*, 2008). The loss of an OMP of 33-36 kDa or OprD-like OMP has been detected in carbapenem-resistant strains (del Mar Tomás *et al*., 2005). This loss is associated with the presence of OXA-51 associated with IS*Aba1* producing the carbapenem-resistant phenotype due to a synergistic effect between both mechanisms (Rumbo *et al*., 2013). Another OMP, CarO, a protein of 29-kDa, has been related with decreased susceptibility to carbapenems (Mussi *et al*., 2007). The expression of CarO is regulated by the activity of IS elements, such as IS*Aba1* and IS*Aba10*, that can disrupt the *carO* gene, interrupting the expression of this OMP (Lee *et al*., 2011; Lu *et al*., 2009). Additionally, over-expression of efflux pumps, specifically ones that belong to the RND family, such as AdeABC (Nemec *et al*., 2007), AdeFGH and AdeIJK (Coyne *et al*., 2010), contribute to the efflux of a broad spectrum of antibiotics, including carbapenems. High levels of resistance are associated with the activity of efflux pumps plus the presence of carbapenemases (reviewed by Evans *et al*., 2013).

1.5.1.3 Epidemiology of MDR-*Acinetobacter baumannii*

As mentioned previously, among the ICs, the IC-II is the most prevalent, where the $bla_{\text{OXA-66}}$ has been frequently detected in Asia and South America (reviewed by Evans *et al*., 2013). Additionally, ICs have been detected recently in strains from Europe, North America and Latin America, most of which were MDR and the majority of them were resistant to

carbapenems (reviewed by Durante-Mangoni and Zarrilli, 2011). In Latin America, few countries possess national surveillance programs for the collection of data of antimicrobial resistance, making it difficult to estimate prevalence and evolution of this phenomena (Okeke *et al*., 2005). In this sense, the SENTRY Antimicrobial Surveillance Program, initiated in 1997, represents the most important source of information about antibiotic resistance in Latin America. According to this programme, *Acinetobacter* spp. causes the 7.2% of the bloodstream infections, the 17.7% of pneumonia, the 9.9% of the skin and soft tissue infections in hospitalised patients between 2008 and 2010 (Gales *et al*., 2012). In this study, both *P. aeruginosa* and *A. baumannii* showed the highest rates of resistance to almost all the drugs available, except to colistin, regardless of the geographic region studied. Importantly, there were significant increases in the imipenem resistance in *Acinetobacter* spp. (Figure 25). Specifically in Chile, Argentina and Brazil, there has been an alarming rise in the rates of imipenem resistance during the last years, rising from 10% or less of resistance between 1997 -1998 to more than 40% in the 2008 - 2010 period (Figure 25).

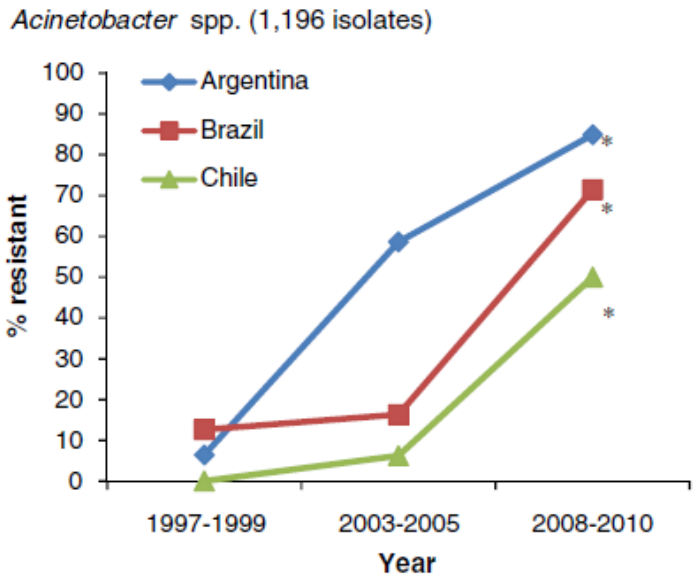

Figure 25. Variation in the imipenem resistance rates overtime in *Acinetobacter* spp. from Latin America (Gales *et al*., 2012).

On the other hand, multiple outbreaks of MDR *A. baumannii* in Asian and Middle Eastern countries have been reported (Peleg *et al.*, 2008). According to the SENTRY Program, the rates of carbapenem non-susceptible isolates are less than 25%, 40% for cefepime and ceftazidime, 40% for the association ampicillin-sulbactam and 45% for ciprofloxacin between 2001 – 2004 (Gales, Jones, & Sader, 2006). Isolates resistant to tigecycline and colistin have been identified in this region (Navon-Venezia *et al*., 2007). According to a study of 2011, the susceptibility of *Acinetobacter* spp. from Tawam Hospital, Al-Ain, UAE; decreased between 2004-2008 to imipenem, ceftazidime, ciprofloxacin, gentamicin and piperacillin/tazobactam, comparing with the period 1999-2002 (Al-Kaabi *et al*., 2011). Specifically, the susceptibility to imipenem decreased from 99.0% in the period 1999-2002 to 32.5% during 2008 and the susceptibility to ceftazidime dropped from 66.3% in 2004 to 31.6% in 2008 (Al-Kaabi *et al*., 2011).

1.6. Aims of this project

Due to the importance of the Mobilome in the acquisition of resistance genes; the objective of this thesis was to investigate the role of plasmids, ISs, transposons-like structures and IS*CR1*-elements in the acquisition of ceftazidime- and carbapenem-resistance in *A. baumannii* from Chile and the UAE.

- To identify the isolates, collected from Chile and the United Arab Emirates between 2007 – 2008, genotypically as *A. baumannii* by amplification of $bla_{\text{OXA-51-like}}$ and *rpoB* genes by the polymerase chain reaction (PCR).

- To characterise the MDR-phenotype and analyse the clonal dissemination of carbapenem-resistant *A. baumannii* isolates collected between 2007 – 2008 from Santiago, Chile and Al Ain, UAE.

- To detect and characterise the carbapenemases genes involved in the carbapenem-resistance in isolates from Chile and the UAE

- To identify mobile genetic elements mediating the carbapenem-resistance in isolates from Chile.

- To characterise the mechanism of ceftazidime-resistance in *A. baumannii* isolates collected in 2008 from Al Ain, UAE.

Chapter 2 Materials and Methods

2.1. Bacterial isolates

Sixteen *A. baumannii* isolates, collected from three different hospitals in Santiago of Chile between 2007 – 2008 and previously classified as carbapenem non-susceptible, were studied. These isolates were provided by Dr. Patricia García (Catholic University Hospital), Dr. Andrea Sakurada (University of Chile Hospital) and Dr. Marcela San Martin (Barros Luco Hospital). The samples were initially identified as *A. baumannii* by phenotypic tests in each individual hospital. Additionally, two *A. baumannii* isolates from Al Ain in the United Arab Emirates, were studied. These samples were provided by Professor Tibor Pál.

For species identification experiments, the type strain *A. baumannii* ATCC 19606 was utilised as a control. For minimum inhibitory concentration tests determinations, the control strains *S. aureus* NCTC 6571, *P. aeruginosa* NCTC 10662, *E. coli* NCTC 10418 and *A. baumannii* ATCC 19606 were used. A list of the strains studied and their epidemiological data is shown in Table 4.

All isolates were sub-cultured on MacConkey agar (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Later, a single colony was transfer into nutrient broth (Oxoid, Basingstoke, UK) and incubated overnight at 37°C with agitation. Afterwards, 100µL of the overnight culture was mixed with 900µL of 100w/w glycerol and store at -80°C.

Table 4. List of isolates studied in this work. H1: Catholic University Hospital; H2: University of Chile Hospital; H3: Barros Luco Hospital. ICU: Intensive care unit; IntCU: Intermediate care unit; ICUs: Intensive care unit (surgical); ICUn: Intensive care unit (neurology); ITU: Intensive therapy unit; CCU: Coronary care unit.

Strain	City/Country	Hospital/ward	Source	Year
Ab1	Santiago/Chile	H1/ICU	Wound	2007
Ab2	Santiago/Chile	H1/ICU	Wound	2007
Ab3	Santiago/Chile	H1/ICU	Urine	2007
Ab4	Santiago/Chile	H1/ICU	Catheter	2007
Ab5	Santiago/Chile	H1/Clinic	Secretion	2007
Ab6	Santiago/Chile	H1/Medicine	Secretion	2007
Ab7	Santiago/Chile	H1/Medicine	Secretion	2007
Ab8	Santiago/Chile	H1/Medicine	Catheter	2007
Ab9	Santiago/Chile	H1/IntCU	Catheter	2007
Ab10	Santiago/Chile	H2/ICUs	Catheter	2008
Ab11	Santiago/Chile	H2/ICU	Wound	2008
Ab12	Santiago/Chile	H2/ICUn	Secretion	2008
Ab13	Santiago/Chile	H3/ICU	Secretion	2008
Ab14	Santiago/Chile	H3/Urgency	Other	2007
Ab15	Santiago/Chile	H3/UTI	Blood	2007
Ab16	Santiago/Chile	H3/Urology	Urine	2007
NM55	Al Ain/UAE	Tawam/CCU	Sputum	2008
NM128	Al Ain/UAE	Tawam/CCU	Sputum	2008

2.2. Media and chemical reagents.

All media and antibiotic discs utilised in this work were purchased from Oxoid (Basingstoke, UK). Broths and agar were prepared following the manufacturer's instructions. They were autoclaved at 121°C/15psi during 15min for sterilisation. The chemical reagents used in this work were purchased from Sigma-Aldrich Company (Poole, UK) unless stated otherwise. The saline solution was prepared by adding 0.8g of NaCl in 100mL of distilled water and autoclaved.

2.3. DNA extraction and PCRs

The isolates were incubated in nutrient broth (Oxoid, Basingstoke, UK) overnight at 37°C with agitation. Later, the culture was transferred into MacConkey agar and incubated at 37°C for 18h. Afterwards, a loopful of bacterial colonies was added to 100µL of sterilised distilled water (SDW) and boiled at 100°C for 10 min. Then, the eppendorf tubes were centrifuged at 13,000rpm (6,000g) for 2min and the supernatant was used as DNA template. For further experiments, the supernatants were stored at 4°C.

All PCRs were performed in total volumes of 50µL. All reagents, except primers that were supplied by Eurofins MWG operon, were purchased from Promega (Southampton, UK). The volumes of each reagent per reactions were: 5µL of 10X buffer (200mM Tris-HCl, 500mM KCl), 1.5µL of 50 mM MgCl2, 1µL of 1mM dNTP mix, 0.5µL of 10µM forward primer, 0.5µL of 10µM reverser primer, 0.25U/µL Taq polymerase, 1µL of DNA template and 40.25 µL of SDW. The primers were either obtained from previously published work or designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Reactions were carried out using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Warrington, UK).

After the PCR amplification, the products were separated by electrophoresis in 1.5w/v agarose gels prepared in 1X TAE buffer (40mM Tris-acetate, pH 8.0, 2mM EDTA). Electrophoresis was performed in horizontal gels units (Bio-Rad, UK) at 100V for 15min. After running, the gels were stained with GelRed stain (Biotium, USA) solution prepared diluting 50µL of GelRed in 100mL of 0.1M NaCl, and visualised using a GelDoc 2000 system (Bio-Rad, Milan, Italy).

For sequencing, the PCR products were purified using the Qiagen purification kit (Qiagen, UK) and 5µL of the purified product was mixed with 1µL of 3.2pmol primers for forward and/or reverse sequencing. The DNA sequencing was performed using an ABI 3730 capillary sequencing instrument (Applied Biosystems, Warrington, UK) by the GenePool laboratory (Ashworth Laboratory, King's Buildings, The University of Edinburgh, UK). The sequences obtained were analysed utilising the BLAST database of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nhi.gov). Alignment of the DNA sequences was carried out with the Multalin software (http://www.toulouse.inra.fr/multalin.html) to check any change in the sequences.

2.4. Species identification

Species identification was carried out by detection of the *bla*$_{\text{OXA-51-like}}$ gene (Hamouda *et al*., 2010; Woodford *et al*., 2006) and by amplification and sequencing of a 350 bp section of the *rpoB* gene (Gundi *et al*., 2009).

The primers used for detecting the *bla*$_{\text{OXA-51-like}}$ genes and the expected size were:

OXA-51-F: 5'-TAA TGC TTT GAT CGG CCT TG-3'

353bp

OXA-51-R: 5'-TGG ATT GCA CTT CAT CTT GG-3'

The set of primers utilised for the detection and sequencing of the 350bp portion of the *rpoB* gene were

rpoB-F: 5'-TAY CGY AAA GAY TTG AAA GAA G-3'
rpoB-R: 5'-CMA CAC CYT TGT TMC CRT GA-3'

Where Y=C, T; M=A, C; R=A, G.

2.5. Disc diffusion tests

Antibiotic susceptibility test were carried out on IST plates agar by the disc diffusion method following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Andrews, 2010, version 9.1) unless otherwise stated. The isolates were classified as susceptible, intermediate or resistant according to the BSAC guidelines. The antibiotics tested with the Chilean isolates were ceftazidime (CAZ), aztreonam (ATM), imipenem (IPM), meropenem (MEM), cefotaxime (CTX), gentamicin (G), amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), cefepime (FEP), piperacillin/tazobactam (TZP) and tigecycline (TGC). The criteria used to analyse the susceptibility to tigecycline, amoxicillin/clavulanic acid, aztreonam and cephalosporins were those proposed by the Clinical and Laboratory Standards Institute (CLSI) (Jones *et al*., 2007). The antibiotic tested with the Emirati isolates were imipenem, meropenem, cefotetan, rifampicin, aztreonam, chloramphenicol, cefoperazone, cefepime, cefotaxime and cefpodoxime. The Emirati isolates were previously tested against tigecycline, ciprofloxacin and gentamicin in the laboratory of Professor Tibor Pál, in the UAE.

2.6. Minimum inhibitory concentrations (MICs) determinations

The Chilean isolates were tested for their susceptibility to imipenem and meropenem. Imipenem was used in its combination with cilastatin with a potency of 50% (Merck, Sharp & Dohme Ltd, Hertfordshire, UK), and meropenem with a potency of 99.8% (AstraZeneca, Cheshire, UK). The isolates were grown on MacConkey agar plates at 37°C for 18h. Later, the isolates were incubated overnight in 5mL of IST-broth at 37°C and 180rpm in an orbital shaker. Afterwards, the MICs were determined on IST agar plates by the double dilution method according to the BSAC recommendations (Andrews, 2010, version 9.1) utilising a Denley multipoint inoculator (Denley, Surrey, UK). For the isolates from the UAE, the same methodology was used exchanging the drugs tested by rifampicin (Sigma-Aldrich, Poole, UK) and ceftazidime (Sigma-Aldrich, Poole, UK).

2.7. Pulsed-field gel electrophoresis (PFGE)

Isolates were typed by PFGE according to Seifert *et al*. (2005) with some modifications.

2.7.1. Plugs preparation

The isolates were inoculated into 5mL of nutrient broth and incubated overnight at 37°C with agitation. Later, the isolates were suspended in to 3mL of suspension buffer (100mM Tris-HCl, 100mM EDTA, pH 8.0). Each sample was adjusted to an absorbance range of 1.7 – 1.8 at 610nm, that represents a cell density of approximately 10^9 cells/mL. Then, 500µL of each suspension were incubated in a water bath at 55°C for 10min. Immediately, 25µL of 10mg/ml proteinase K, 500µL of melted 1% CHEF genomic agarose (Bio-Rad, UK) and 1% of sodium dodecyl sulphate (Fisher Scientific, UK) dissolved in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) were added to the suspensions and they were mixed by inverting the

tubes ten times. Later, the samples were transferred in to plastic moulds (Bio-Rad, UK) and were allowed to solidify at 4°C during 5min.

2.7.2. Cell lysis

The plugs were extracted from the moulds and were placed into 50mL plastic tubes containing 5mL of lysis buffer (50mM Tris, 50mM EDTA, pH 8.0, 1% sarcosine) and 25µL of proteinase K (20mg/ml stock solution). Then, the samples were incubated for 2h at 55°C in a water bath. Later, the lysis buffer was removed and the samples were washed two times with SDW and three times with TE buffer for 15min/wash at 55°C in a water bath.

2.7.3. Enzymatic restriction and electrophoresis

A slice of each plug (4mm approximately) was incubated with 10µL of 10X buffer A (Promega, Southampton, UK) and 90µL of SDW for 15min at 25°C in a water bath. Later, the reaction buffer was removed and replaced by enzymatic digestion buffer comprising 88µL of SDW, 9µL of 10X buffer A and 30U of *Apa*I (Promega, Southampton, UK) restriction enzyme and each sample was incubated at 25°C for 18h. The enzymatic reaction was stopped by removing the enzymatic digestion buffer and adding 100µL of 0.5X TBE buffer (0.89M Tris, 0.89M boric acid, 20mM EDTA, pH 8.0). Afterward, the plugs were loaded on a 1% CHEF agarose gel (Bio-Rad, UK) dissolved in to 90mL of 0.5X TBE buffer and the electrophoresis was run on a CHEF-DRII apparatus (Bio-Rad, UK) at 6V/cm and 14°C during 19h with switch times ranging from 5s to 20s. After the running, the gel was stained for 30min with 200mL of GelRed solution and destained for 15min with SDW.

2.7.4. Data analysis

The gels were visualised using a GelDoc 2000 system (Bio-Rad, Milan, Italy) and the images were analysed by the BioNumerics v7.0 software (Applied Maths, Sint-Martens-Latem, Belgium). The clusters analysis was performed by the unweighted pair group (UPGMA) method using a Dice coefficient of 1.5% for each band and optimization of 1.5%. A grouping percentage of ≥87% was chosen as the threshold to establish clonal relatedness.

2.8. Detection of β-lactamases genes

Detection of *bla*$_{OXA}$ genes was carried out by PCRs. All primers utilised are listed in Table 5.

Table 5. Primers used for detecting *bla*$_{OXA\text{-}like}$ genes. [1]Woodford *et al*., 2006; [2]Higgins *et al*., 2013; [3]Héritier *et al.*, 2005a.

Primers	Sequence (5' → 3')	Product size
OXA-23-F[1]	GATCGGATTGGAGAACCAGA	501bp
OXA-23-R[1]	ATTTCTGACCGCATTTCCAT	
OXA-24-F[1]	GGTTAGTTGGCCCCCTTAAA	246bp
OXA-24-R[1]	AGTTGAGCGAAAAGGGGATT	
OXA-51-F[1]	TAATGCTTTGATCGGCCTTG	353bp
OXA-51-R[1]	TGGATTGCACTTCATCTTGG	
OXA-58-F[1]	AAGTATTGGGGCTTGTGCTG	599bp
OXA-58-R[1]	CCCCTCTGCGCTCTACATAC	
OXA-143-F	TTCTGTCAGTGCATGCTCATC	728bp
OXA-143-R	CAGGCATTCCTTGCTTCATT	
OXA-235-F[2]	TTGTTGCCTTTACTTAGTTGC	768bp
OXA-235-R[2]	CAAAATTTTAAGACGGATCG	
OXA69-A[3]	CTAATAATTGATCTACTCAAG	No IS upstream → 975bp
OXA69-B[3]	CCAGTGGATGGATGGATAGATTATC	IS element upstream → variable

Detection of the groups of *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$ and *bla*$_{\text{OXA-58-like}}$ genes was carried out using the primers listed on Table 5 according to Woodford *et al* (2006). Detection and sequencing of *bla*$_{\text{OXA-51-like}}$ genes was performed using the primers and conditions described by Héritier *at al*. (2005a). The pair of primers utilised for this PCR allowed the detection of IS elements inserted upstream the *bla*$_{\text{OXA-like}}$ genes. Detection of *bla*$_{\text{OXA-235}}$ was performed according to Higgins *et al*. (2013).

Detection of *bla*$_{\text{OXA-143-like}}$ group was carried out using the primers listed on Table 5 using the following conditions:

Pre-denaturation	94°C for 5min	1 cycle
Denaturation	94°C for 30s	
Annealing:	57°C for 40s	30 cycles
Extension:	72°C for 50s	
Final extension:	72°C for 6min	1 cycle
Cooling:	4°C	

Detection of *bla*$_{ADC}$, *bla*$_{PER\text{-}like}$, *bla*$_{VEB\text{-}like}$, *bla*$_{GES\text{-}like}$ and *bla*$_{NDM\text{-}like}$ genes was carried out using the following primers:

Table 6. Primers used for detecting *bla*$_{ADC}$, ESBLs and *bla*$_{NDM\text{-}like}$ genes. [1]Ruiz *et al*., 2007a.

Primers	Sequence (5' → 3')	Product size
ADC1[1]	CCGCGACAGCAGGTGGATA	420bp
ADC2[1]	TCGGCTGATTTTCTTGGTT	
PER-F	CCTGACGATCTGGAACCTTT	715bp
PER-R	GCAACCTGCGCAATGATAGC	
VEB-F	ATTTCCCGATGCAAAGCGT	362bp
VEB-R	CCAACAGCGATGAACAAACT	
GES-F	ATGCGCTTCATTCACGCAC	858bp
GES-R	AACTCATCCTGAGCACGGAC	
NDM-F	GGGCCGTATGAGTGATTG	535bp
NDM-R	GCACACTTCCTATCTCGAC	

Detection of *bla*$_{ADC}$ genes was carried out according to Ruiz *et al*. (2007a). Detection of ESBLs and *bla*$_{NDM}$ was performed using the primers listed on Table 6 and under the following conditions:

Pre-denaturation	94°C for 5min	1 cycle
Denaturation	94°C for 30s	
Annealing:	57°C for 40s	35 cycles
Extension:	72°C for 50s	
Final extension:	72°C for 6min	1 cycle
Cooling:	4°C	

2.9. Detection of Insertion Sequences (ISs) and IS*CR*-like elements.

PCR amplification follow by sequencing were performed in order to analyse the genetic environment of the *bla*$_{\text{OXA-58}}$ genes in the Chilean isolates. For this purpose, the primers utilised were SM2 (5'-AAGTGTCTATATTCTCACC-3') (Poirel and Nordmann, 2006) in combination with the OXA-58-R primer. The SM2 forward primer aligns in an intergenic region upstream the *bla*$_{\text{OXA-58}}$ genes, allowing the detection of any insertion element related with these genes. To analyse the downstream region, a PCR was performed utilising the OXA-58-F primer and the IS*Aba3*-R primer (5'-ATGCTGCCTTTAAATGAAGCCA-3').

The PCRs were carried out under the following conditions:

Pre-denaturation	94°C for 5min	1 cycle
Denaturation	94°C for 30s	
Annealing:	55°C for 40s	35 cycles
Extension:	72°C for 50s	
Final extension:	72°C for 6min	1 cycle
Cooling:	4°C	

To detect and characterise the IS*CR1* element, which carried the *bla*$_{\text{PER-like}}$ gene in the isolates from the UAE, PCR and sequencing were performed. The detection and characterisation of the integron class 1 portion of the IS*CR1* element was performed as previously described (Bae *et al*., 2007).

The detection of the *orf513* gene was carried out by PCR using the following primers (Opazo *et al*., 2012a):

ORF513-F:	5'-TCAAAGAGACGACTCTGTGATGGAT-3'	1022bp
ORF513-R:	5'-TGACTCTTATCCAACGCTTTGGC-3'	

A set of PCRs and sequencing were utilised in order to investigate the genetic environment and the sequence of the $bla_{\text{PER-like}}$ gene. The different combinations of primers were: ORF513-F with PER-R; PER-F with gst-R (5'-GTTCAGTGGCTTCCCCTTTT-3') and gst-F (5'-GAACGGCCTTCAGACTCAAA-3') with Abau-R (5'-GGGTTTCCGAGAAGGTGATT-3') (Opazo *et al*., 2012a).

All PCRs were performed under the following conditions:

Pre-denaturation	94°C for 5min	1 cycle
Denaturation	94°C for 30s	
Annealing:	57°C for 40s	35 cycles
Extension:	72°C for 50s	
Final extension:	72°C for 6min	1 cycle
Cooling:	4°C	

In order to investigate the association between the *bla*$_{ADC}$ genes and insertion sequences upstream of them, a standard PCR and sequencing were performed using the following primers:

FU: 5'- GCGCCGTGAATTCTTAAGTG-3'

RU: 5'- AGCCATACCTGGCACATCAT-3'

360bp

The expected size, when there were no insertion elements upstream, was 360bp. On the other hand, if any insertion sequence was located upstream the *bla*$_{ADC}$ gene, the product size varied, but was somewhat larger that 360bp.

The PCR conditions were:

Pre-denaturation	94°C for 5min	1 cycle
Denaturation	94°C for 30s	
Annealing:	57°C for 40s	35 cycles
Extension:	72°C for 50s	
Final extension:	72°C for 6min	1 cycle
Cooling:	4°C	

2.10. Plasmid analysis

In order to analyse the presence of the *bla*$_{OXA-58}$ gene in plasmids in the Chilean strains, the plasmids were extracted using a commercial kit (Promega, UK). After the extraction, the plasmids were separate by PFGE at 6V/cm and 14°C during 20h with an initial pulse of 5s and a final pulse of 20s. Afterwards, the gels were stained in a GelRed solution for 30min and destained for 15min with SDW. Later, the plasmid bands were extracted from the gel and the plasmid DNA was purified using the Qiagen extraction kit (Qiagen, UK). The plasmid DNA extracted was used as a DNA template for PCR experiments. In order to detect any chromosomal contamination, a PCR using the 16S-rRNA primers previously described (Lin *et al*., 2009) was utilised. To determine whether the *bla*$_{OXA-58}$ genes were plasmid-located, standard PCRs using the primers previously described in this section, were utilised.

2.11. Gene expression analysis

2.11.1. RNA extraction

The expression of the *bla*$_{OXA-58}$ genes was studied by RT-PCR. The bacterial RNA was obtained by inoculating the isolates into nutrient broth and they were grown to an optical density of 0.5 – 0.8 at 600 nm. Later, the RNA was extracted with the RiboPureTM-Bacteria kit following the manufacturer's recommendations (Ambion, UK). In order to avoid any contamination, the benches and gloves were sterilised using the RNAZap solution (Ambion, UK). After the extraction, the concentration of RNA was measured using a NanoDrop spectrophotometer, ND-1000. Finally, any DNA contamination was eliminated by incubating the samples with DNAse I (Ambion, UK) at 37°C for 30min.

2.11.2. Reverse-transcription PCR (RT-PCR) and data analysis

To analyse the expression of the *bla*$_{\text{OXA-58}}$ gene, 100ng of the purified RNA were reverse transcribed in to cDNA and a PCR detecting the *bla*$_{\text{OXA-58}}$ was performed using the Access RT-PCR System Kit (Promega, UK), using the primers described earlier in this section. After the PCR program, the samples were separated by electrophoresis and the band intensity was quantified using the Bio-Rad Quantity One software version 4.6.1.

3.1. To identify the isolates, collected from Chile and the United Arab Emirates between 2007 – 2008, genotypically as *A. baumannii* by amplification of *bla*$_{\text{OXA-51-like}}$ and *rpoB* genes by the polymerase chain reaction (PCR).

The Chilean isolates were primarily identified as *A. baumannii* by detecting the *bla*$_{\text{OXA-51-like}}$ genes by standard PCR (Evans *et al*., 2008). All the isolates from Chile, except Ab11, were positive for this gene. An example gel showing the amplification of the *bla*$_{\text{OXA-51-like}}$ genes is shown in Figure 26.

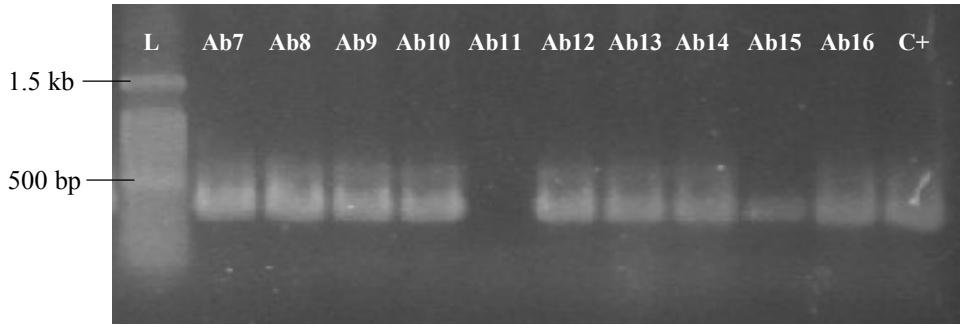

Figure 26. Example gel of amplification of the *bla*$_{\text{OXA-51-like}}$ genes by PCR. The isolate Ab11 was negative for the detection of *bla*$_{\text{OXA-51-like}}$. C+: positive control *A. baumannii* ATCC 19606; L: 100bp DNA ladder.

As shown in Figure 26, the isolate Ab11 was negative for the amplification of the *bla*$_{\text{OXA-51-like}}$ gene, hence this isolate was discarded for further analysis. The PCR was validated using the strain type *A. baumannii* ATCC 19606 as a positive control. All the Chilean isolates were previously phenotypically identified as *A. baumannii* in each particular hospital, however, my result shows that the isolate Ab11 had been erroneously classified, which represents a problem for hospitals as they do not have a routine molecular identification protocol, and

probably the real number of infections caused by *A. baumannii* is over-estimated. Therefore, it is necessary to implement new methods of species identification as the cases of outbreaks caused by MDR *A. baumannii* have been increasing during recent years, necessitating an accurate classification of this microorganism.

In the case of the samples from the United Arab Emirates, they were previously classified as *A. baumannii* and they were primarily confirmed as such by detection the *bla*$_{\text{OXA-51-like}}$ genes, as shown in the Figure 27.

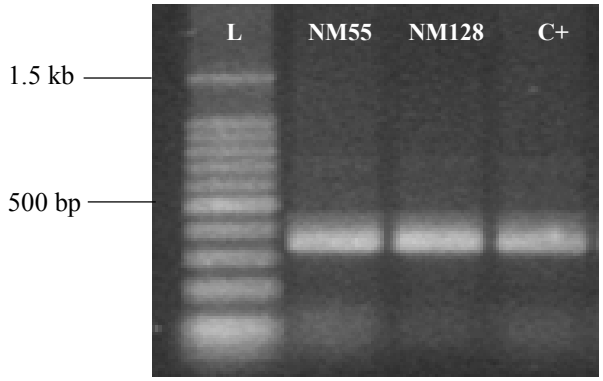

Figure 27. Detection of the *bla*$_{\text{OXA-51-like}}$ genes in the isolates from the UAE. C+: positive control *A. baumannii* ATCC 19606; L: 100bp DNA ladder.

In summary, all the samples from the United Arab Emirates and 15 samples from Chile were initially identified as *Acinetobacter baumannii*, discarding the isolate Ab11 as it had not shown a band corresponding to the *bla*$_{\text{OXA-51-like}}$ genes.

The amplification of the *bla*$_{\text{OXA-51-like}}$ genes has been considered a simple, rapid and reliable method for species identification as these are naturally occurring carbapenemases genes to *A. baumannii* (Turton *et al*., 2006b). Strains of *A. baumannii*, which are carbapenem

susceptible also carry these carbapenemases genes, as they confer resistance to carbapenems only when a insertion element, such as IS*Aba1*, is located upstream (Turton *et al*., 2006a), leading to overexpression of the gene.

Even though the detection of the *bla*$_{\text{OXA-51-like}}$ genes has been considered a rapid and reliable method to identify isolates as *A. baumannii*, the detection of these genes only is not enough to conclude that an isolate corresponds to this species. In this regard, in 2013, Zander *et al* demonstrated that in three *A. baumannii* isolates obtained from South Korea, South Africa and Turkey, the amplification of the *bla*$_{\text{OXA-51-like}}$ genes was negative for the expected PCR product of 353bp as in the multiplex-PCR proposed by Woodford *et al* (2006). However, the samples were positive for larger amplicons when the *bla*$_{\text{OXA-51-like}}$ genes were amplified by the multiplex-PCR described earlier. Besides, the samples were classified as carbapenem susceptible, therefore the variation of the amplicon sizes was not due the presence of an IS element upstream the genes. The amplicons sizes varied from 1.2 to 1.6kb. After the sequencing of the PCR products, it was possible to identify the presence of two insertion sequences inserted within the *bla*$_{\text{OXA-51-like}}$ genes, which generate the change of the size of the amplification products. The analysis of the complete sequence revealed the presence of the *bla*$_{\text{OXA-66}}$ gene interrupted by the IS*Aba15* and the presence of the *bla*$_{\text{OXA-51-like}}$ variant *bla*$_{\text{OXA-78}}$ disrupted by IS*Aba19* (Zander *et al*., 2013). In the case of the isolate Ab11 studied in this work, it did not show any amplification product, therefore the absence of a *bla*$_{\text{OXA-51-like}}$ gene and/or a variant disrupted by an IS element determined that it was discarded.

Besides the variation of the structure of the *bla*$_{\text{OXA-51-like}}$ genes due the presence of insertion elements, some *bla*$_{\text{OXA-51-like}}$ variants have been detected in plasmids from *Acinetobacter* species other than *A. baumannii*. Specifically, Lee *et al*. (2012) studied the presence of *bla*$_{\text{OXA-51-like}}$ variants in *A. nosocomialis* and *Acinetobacter* genomic species "close to 13TU"

which were not susceptible to carbapenems. All the isolates studied harboured the association IS*Aba1*-*bla*$_{\text{OXA-51-like}}$. The *bla*$_{\text{OXA-51-like}}$ variants identified were *bla*$_{\text{OXA-194}}$ and *bla*$_{\text{OXA-138}}$. Interestingly, a similar genetic structure harbouring the plasmid-borne *bla*$_{\text{OXA-82}}$ variant was found in an carbapenem-resistant *A. baumannii* isolate from Taiwan (Chen *et al.*, 2010). Comparing the plasmids that carry the *bla*$_{\text{OXA-51-like}}$ genes in *A. baumannii* with the plasmids present in *A. nosocomialis* and *Acinetobacter* genomic species "close to 13TU", the plasmid sizes were similar, at 50kb. This finding indicates that the dissemination of these genes is probably either due by the mobilisation of this specific plasmid from different *A. baumannii* strains or the plasmids were disseminated among different clones of non-*Acinetobacter baumannii* species or even this dissemination is due the clonal dissemination of *Acinetobacter* species other than *A. baumannii*.

Due the fact that these genes can be found on plasmids from species other than *A. baumannii*, the detection of the *bla*$_{\text{OXA-51-like}}$ genes as a unique method for species confirmation is insufficient for species identification, making it necessary to use a complementary method in order to ensure that the isolates studied correspond to *A. baumannii*. Other secondary methods include *gyrB* multiplex PCR, amplification and sequencing of the *rpoB* gene, tRNA spacer fingerprinting, sequencing of the 16S rDNA, PCR analysis of the *recA* gene, sequencing of the intergenic rDNA region, among others (Evans *et al*., 2013).

In order to ensure that the isolates corresponded to *A. baumannii*, species identification was confirmed by a second methodology, specifically by amplification and sequencing of a 350bp portion of the *rpoB* gene (Gundi *et al*., 2009).

All the isolates analysed were positive for the 350bp amplicon. The sequences obtained showed a similarity of 99% with the sequence of the *rpoB* gene available in the NCBI database (Figure 28) (accession number HQ123410.2).


Download GenBank Graphics

Acinetobacter baumannii partial rpoB gene for RNA polymerase beta-subunit, strain 267

Sequence ID: emb|HG515013.1| Length: 341 Number of Matches: 1

Range 1: 20 to 341 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
595 bits(322)	8e-167	322/322(100%)	0/322(0%)	Plus/Plus

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Query  1    TTCGTTTGCTTAAAGGCCAAGAGTCTAATGGCGGTGGTTCAACTAAACGTGGTGATAAAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20   TTCGTTTGCTTAAAGGCCAAGAGTCTAATGGCGGTGGTTCAACTAAACGTGGTGATAAAC  79

Query  61   TTTCTGAAGATTTATTATCTGGTTTAGAGCTTGTTGACTTACTTGAAATTCAACCAGCAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80   TTTCTGAAGATTTATTATCTGGTTTAGAGCTTGTTGACTTACTTGAAATTCAACCAGCAG  139

Query  121  ATGAAGCAATCGCTGAGCGTTTAACTCAAATTCAAGTGTTCTTGAAAGAGAAGAGCGCAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  ATGAAGCAATCGCTGAGCGTTTAACTCAAATTCAAGTGTTCTTGAAAGAGAAGAGCGCAG  199

Query  181  AAATCGATGAGAAATTCGCTGAGAAGAAACGTAAGCTTGCAACAGGTGATGAATTAACAA  240
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Query  301  ATAAGATGGCTGGTCGTCACGG  322
            |||||||||||||||||||||
Sbjct  320  ATAAGATGGCTGGTCGTCACGG  341
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Figure 28. Comparison of the *rpoB* gene of the Ab1 isolate with the sequence available in the NCBI database. Query: Nucleotide sequence of *rpoB* of isolate Ab1; Sbjct: Subject sequence in the NCBI database.

The *rpoB* gene, which encodes an RNA polymerase β-subunit, possesses four hypervariable zones. Among these different regions, the highly variable zone of 350bp (zone 1) differs between closely related *Acinetobacter* species. Specifically, according to Gundi *et al*. (2009), zone 1 shows a similarity range of 88.3%-96.9% when *A. baumannii*, *A. pittii* and *A. nosocomialis,* are compared. This similarity range increases to 98%-100% when the comparison is intraspecies. Due to these characteristics, the amplification and sequencing of

the *rpoB* gene represents a reliable and rapid method for species identification, which can be complemented, with other methods, such as detection of the $bla_{\text{OXA-51-like}}$ gene.

As all the samples analysed in this work for sequencing of the 350bp portion of the *rpoB* shared more than 99% of similarity with the sequence available in the NCBI database, consequently they were classified as *A. baumannii* species.

3.2. To characterise the MDR-phenotype and analyse the clonal dissemination of carbapenem-resistant *A. baumannii* isolates collected between 2007 – 2008 from Santiago, Chile and Al Ain, UAE.

3.2.1. Susceptibility tests

The isolates were tested by the disc diffusion method against different classes of antibiotics including carbapemens, cephalosporins, aminoglycosides, penicillins, fluoroquinolones and glycylcyclines.

3.2.1.1. Chilean isolates

All the Chilean isolates were resistant to ceftazidime, aztreonam, imipenem, cefotaxime, gentamicin, amoxicillin/clavulanic acid, meropenem, ciprofloxacin and cefepime (Figure 29).

Isolate Ab13, isolated from the Barros Luco Hospital, was the only isolate susceptible to the piperacillin/tazobactam combination. The rates of nonsusceptibility to piperacillin/tazobactam are one of the highest in the world (Peleg *et al*., 2008). According to the SENTRY Program, the percentage of susceptibility to piperacillin/tazobactam in Chilean isolates is 13.9% for the 2008-2010 period (Gales *et al*., 2012). The effectiveness of this antibiotic combination has been decreasing as the percentage of susceptibility to this drug in *A. baumannii* from Latin America was 27.7% in 2001 (Diomedi, 2005).

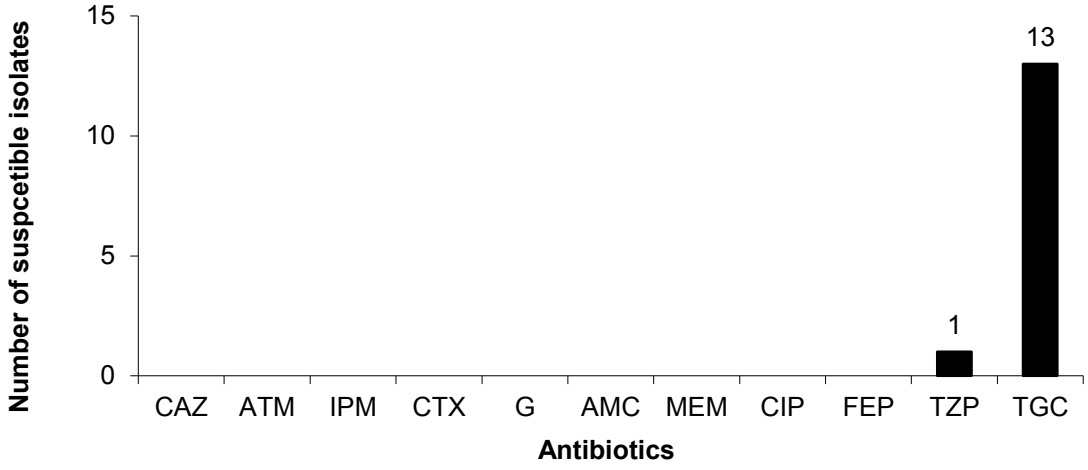


Figure 29. Disc diffusion test results for Chilean isolates (n=15). CAZ: ceftazidime; ATM: aztreonam; IPM: imipenem; CTX: cefotaxime; G: gentamicin; AMC: amoxicillin/clavulanic acid; MEM: meropenem; CIP: ciprofloxacin; FEP: cefepime; TZP: piperacillin/tazobactam; TGC: tigecycline.

All the Chilean isolates were resistant to the cephalosporins tested (cefotaxime, cefepime and ceftazidime) (Figure 29). Specifically, the resistance to ceftazidime in Chilean isolates of *Acinetobacter* spp. was one of the highest in comparison with other Latin American countries during the 2008-2010 period (Gales *et al*., 2012). The susceptibility of *A. baumannii* to these drugs, as in the case of the majority of the other antibiotics, has been varying during the recent years. Specifically, the percentage of susceptibility to ceftazidime in isolates collected in Latin America, reached 29.0% in 1997, rising to 45.8% in 2001, showing an improvement in the activity of this antibiotic (Diomedi, 2005). However, during the 2008-2010 period in Chile, the susceptibility to this drug decreased to 15.2%, being less effective against *A. baumannii* (Gales *et al*., 2012). The isolates analysed follow this trend, as ceftazidime and other cephalosporins tested do not have any activity against them. Cephalosporins represents an important option to treat infections caused by this microorganism, therefore is important to characterise the most prevalent mechanisms of

resistance present in a specific geographic area. There are currently no studies oriented to analyse the mechanisms of cephalosporin-resistance in Chile.

Furthermore, the samples were non-susceptible to the amoxicillin/clavulanic acid. There are no studies analysing the prevalence and mechanisms of resistance to this antibiotic combination in *A. baumannii* from Chile. Additionally, all the Chilean isolates were non-susceptible to aztreonam. This drug can be an important alternative to treat infections caused by carbapenem-resistant *A. baumannii* as the antibacterial activity of aztreonam increases when is used in combination with polyamines, such as spermine and spermidine (Malone and Kwon, 2013). Specifically, the MICs of aztreonam alone (≥128 mg/L) decreased to the range 0.25-8 mg/L when was used in combination with polyamines. Importantly, this effect occurred when polyamines were used in combination with aztreonam and other β-lactams except ceftazidime and meropenem (Malone and Kwon, 2013). Even though the mechanism of action of polyamines is unknown, it has been suggested that as these molecules are cationic compounds, they can interact with the negative outer membrane of *A. baumannii*, increasing the permeability to aztreonam (Malone and Kwon, 2013). Although this is a recent study, this strategy can represent a valuable alternative to carbapenems.

The isolates from Chile were resistant to gentamicin (Figure 29). According to the SENTRY Program, during the period 2008-2010, the percentage of susceptibility of *A. baumannii* to this drug was 41.8% in Chile (Gales *et al*., 2012). In a study that analysed a set of 487 isolates of *A. baumannii* from Chile collected during the 1993-1994 period, the percentage of resistance to gentamicin reached 94% (Gonzalez *et al*., 2000), indicating an important reduction of the activity of this drug.

Additionally in a study conducted by the SENTRY Program, the percentage of susceptibility to gentamicin in *A. baumannii* from Latin America was 30.7% during 2001 (Tognim *et al.*, 2004) whereas according to a report of the Chilean Society of Infectology (2012), the percentage of susceptibility of *A. baumannii* to gentamicin, was 48.7% in hospitalised patients. This percentage is higher than the figure informed in the SENTRY Program for the period 2008-2010, which was 41.8% (Gales *et al*., 2012). These differences might be because the study performed by the Chilean Society of Infectology included more samples (2135) and more hospitals (28) than the study of the SENTRY Program, which included 79 isolates and 2 hospitals from Santiago, therefore, these results are more representative that those informed by the SENTRY Program. As showed previously, there are variations in the percentages of susceptibility to gentamicin, which makes necessary to perform more studies including more isolates and more medical centres through the country.

The isolates collected from Chile were resistant to ciprofloxacin (Figure 29). The percentage of susceptibility, according to the SENTRY Program, to this fluoroquinolone in *A. baumannii* from Chile was 16.5% during the 2008-2010 period (Gales *et al*., 2012). Previously, the susceptibility to ciprofloxacin in *A. baumannii* from Latin America was 28.3% in 2001 (Tognim *et al*., 2004). Additionally, in a larger study that included isolates collected between January and December 2012, the percentage of ciprofloxacin susceptibility in samples obtained from hospitalised patients from 28 Chilean medical centres was 22.9% (report of the Chilean Society of Infectology, 2012, unpublished). Compared with the 2008-2010 period, the isolates from Chile were less susceptible to ciprofloxacin, thus reducing the therapeutic options to treat infections caused by *A. baumannii*.

Interestingly, Ab14 and Ab16 were not susceptible to tigecycline, as their inhibition zone diameters varied between 16 and 15mm, respectively. The criteria used to interpret the susceptibility to tigecycline is the one proposed by the CLSI (Jones *et al*., 2007) which considers an isolate as susceptible when the inhibition zone is ≥19mm, intermediate between 15-18mm and resistant when the zone diameter is ≤14mm, whence the isolates were classified as intermediate.

The report of the 2012 SENTRY Program did not included this antibacterial agent in its analysis. However, according to a report of the Chilean Society of Infectology from 2009, which included 840 isolates from Chilean hospitals, the percentage of susceptibility to tigecycline was 94.8%, representing the second most active drug against *A. baumannii* after colistin during this period (Silva *et al*., 2011). The susceptibility to tigecycline in Chile has varied dramatically after 2009 as according to a newer study carried out by the Chilean Society of Infectology in 2012, the percentage of susceptibility to tigecycline fell to 62.7% in *A. baumannii* collected from hospitalised patients (report of the Chilean Society of Infectology, 2012, unpublished). This variation may be due to the increase in the usage of this drug in the country as it represents a potent option to treat complicated infections caused by MDR *A. baumannii*. This marked difference in susceptibility values to tigecycline may be also due to the variability of the methods utilised in this study, making it necessary to standardize the methods employed among different centres.

The Chilean isolates were non-susceptible to carbapenems (Figure 29). In accordance to the report of the SENTRY Program of 2001, the percentage of susceptibility to meropenem in *A. baumannii* from Latin America was 81.6% while to imipenem was 83.7% (Tognim *et al*., 2004). However, in a new report of the SENTRY Program published in 2012 the

susceptibility to carbapenems in isolates from Chile decreased dramatically. Specifically, the susceptibility to imipenem was 47.4% and to meropenem was 46.8% (Gales *et al*., 2012). The imipenem-resistance has been significantly changing in Chile, while during the 1997-1999 period, the imipenem-resistance was 0.0%, changing during the 2003-2005 period, when the imipenem-resistance was 6.2% and finally the rates of imipenem-resistance rose to 50.0% during 2010-2012 (Gales *et al*., 2012). Nevertheless, the limitation of the SENTRY Program study of 2012, as in the case of other figures described above, is the number of medical centres and isolates analysed. In the report of the Chilean Society of Infectology of 2012, which included a larger number of samples (2135) and hospitals (23), the susceptibility to imipenem in *A. baumannii* from Chile was 32.3% and to meropenem was 26.9% (report of the Chilean Society of Infectology, 2012, unpublished). The rapid changes in the susceptibility to carbapenems in Chile represent a problem to treat infections caused by this microorganism. Even though the trend of carbapenem-susceptibility is clear in the country. It is necessary to get more information about this, including the study of more medical centres and more isolates. This is because the evolution of the carbapenem-resistance is clearly rapid which makes it necessary to take immediate action, otherwise these potent drugs will become useless within a few years.

In conclusion, the samples collected from Chile were classified as MDR as they were resistant to antipseudomonal cephalosporins (cefepime and ceftazidime), carbapenems (imipenem and meropenem), fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin), which represents the raise of MDR isolates from Chile during recent years.

The MICs to carbapenems of the Chilean isolates to carbapenems were determined by the double dilution method according to the BSAC's recommendations. The results are shown in the Table 7.

According to the BSAC guidelines (Andrews, 2010), the MIC breakpoints to carbapenem are >8mg/L for resistant strains, between 4-8 for intermediate and ≤2mg/L for susceptible. The Chilean isolates analysed were categorised as imipenem-resistant, except the isolates Ab9 and Ab15, which had MICs of 8mg/L, corresponding to intermediate. In the case of meropenem, the isolates Ab1, Ab2, Ab3 and Ab16 had MICs values higher than 8mg/L; in consequence, they were classified as meropenem-resistant. The remaining isolates were intermediate to meropenem (Table 7), as they MICs values to this carbapenem oscillated between 4 and 8mg/L.

The MIC results showed a correlation between the disc diffusion tests performed and the double dilution diffusion test, as both methods classified the isolates as carbapenem-non-susceptible. It is important to analyse the evolution of the carbapenem-resistance in Chile, since the number of isolates resistant to this potent class of antibiotics during recent years in this country has been increasing. Moreover, it is highly important to identify the genes involved in the carbapenem-resistant from this country. In this sense, according to the SENTRY Program (Gales *et al*., 2012), the most prevalent OXA-type carbapenemase gene in South America during 2008-2010 was the *bla*$_{\text{OXA-23-like}}$ group, specifically in isolates from Argentina and Brazil. However, in the specific case of Chile, this trend was different, as the *bla*$_{\text{OXA-58-like}}$ group was the most prevalent (Gales *et al*., 2012).

Table 7. Minimum inhibitory concentrations (MICs) to carbapenems of *A. baumannii* isolates from Chile.

Strain	Imipenem MIC (mg/L)	Meropenem MIC (mg/L)
Ab1	16	32
Ab2	16	32
Ab3	16	32
Ab4	16	8
Ab5	16	8
Ab6	16	8
Ab7	16	8
Ab8	16	8
Ab9	8	4
Ab10	32	8
Ab12	32	8
Ab13	32	8
Ab14	32	8
Ab15	8	16
Ab16	32	4

3.2.1.2. Emirati isolates

According to the disc diffusion method, the isolates from the UAE were resistant to imipenem, meropenem, cefotetan, aztreonam, cefoperazone, cefepime, cefotaxime and cefpodoxime. However, the NM55 isolate was also resistant to rifampicin and ceftazidime, while the NM128 isolate was susceptible to both antibiotics. Both samples were previously characterised in the laboratory of Professor Tibor Pál in the UAE as ciprofloxacin and gentamicin resistant and, in turn, they were were tigecycline-susceptible. Thus, this drug represents an important option to treat infections caused by this microorganism. According

to the definition of MDR proposed by Peleg *et al.* (2008), the Emirati strains were classified as MDR-*A. baumannii* as they were resistant to antipseudomonal carbapenems (imipenem and meropenem), fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin).

In the UAE, as in the rest of the world, the susceptibility rates to different classes of antibiotics decreased dramatically, specifically from the 1999-2002 period to the period 2004-2008 (Al-Kaabi *et al*., 2011), when NM55 and NM128 were collected. It is necessary to perform more studies analysing the trends of antibiotic-resistance in the country, as there is a lack of national data, which is a key factor to improve the usage of antimicrobials.

The difference in the ceftazidime-resistance between both strains was crucial and important to investigate, as they were collected from the same patient 4 months apart. This difference between them may be due to the ability of *A. baumannii* to develop resistance to all types of antibiotics. In this sense, the susceptibility to ceftazidime in 2004 was 66.3%, and dropped to 31.6% during 2008 in Tawam Hospital (Al-Kaabi *et al*., 2011). This change could be due to the appearance of mechanisms of resistance that have not previously been identified in the UAE, such as production of uncommon ESBLs.

The MICs of ceftazidime were 64.0mg/L for isolate NM55 and 0.2mg/L for isolate NM128. Moreover, the MICs of rifampicin were 32.0mg/L for isolate NM55 and 2.0mg/L for isolate NM128. Interestingly, the isolates were collected consecutively from the same patient 4 months apart, showing an important difference in the pattern of ceftazidime-resistance, particularly as the more resistant isolate NM55 was collected first. As mentioned previously, the main mechanism of ceftazidime-resistance is the overexpression of the $bla_{\text{ADC-like}}$ genes driven by the presence of IS*Aba1* upstream (Ruiz *et al*., 2007a). However, this element was

not present upstream the *bla*$_{\text{ADC-like}}$ gene in both isolates, which had previously been determined by the laboratory of Professor Tibor Pál in the UAE and reconfirmed by myself, suggesting the presence of other, less common mechanisms of resistance. This could include the presence of ESBLs, the investigation of which formed the next objective of this thesis.

The first sample (NM55) collected was resistant to ceftazidime, while the isolate collected later (NM128) was susceptible to this antibiotic, reflecting the ability to this microorganism to catch and/or lose genetic elements rapidly. The resistance to rifampicin also differed between both isolates, as the NM55 isolate was resistant to this drug whereas NM128 was susceptible. The mechanism of rifampicin-resistance that may explain this difference was investigated and the results are shown in the next objective of this thesis. The change in the susceptibility patterns of ceftazidime and rifampicin may be due to a change in the therapy plan, to which the patient was probably subjected.

3.2.2. Clonal distribution of *A. baumannii*

The Chilean isolates were analysed by PFGE in order establish their clonal relationship. The geographic distribution of the three hospitals analysed is shown in the Figure 30. The Catholic University Hospital (H1) corresponds to a private medical centre, whereas the other two hospitals analysed (H2 and H3) are public centres and process a higher number of patients than the Catholic University Hospital (H1).

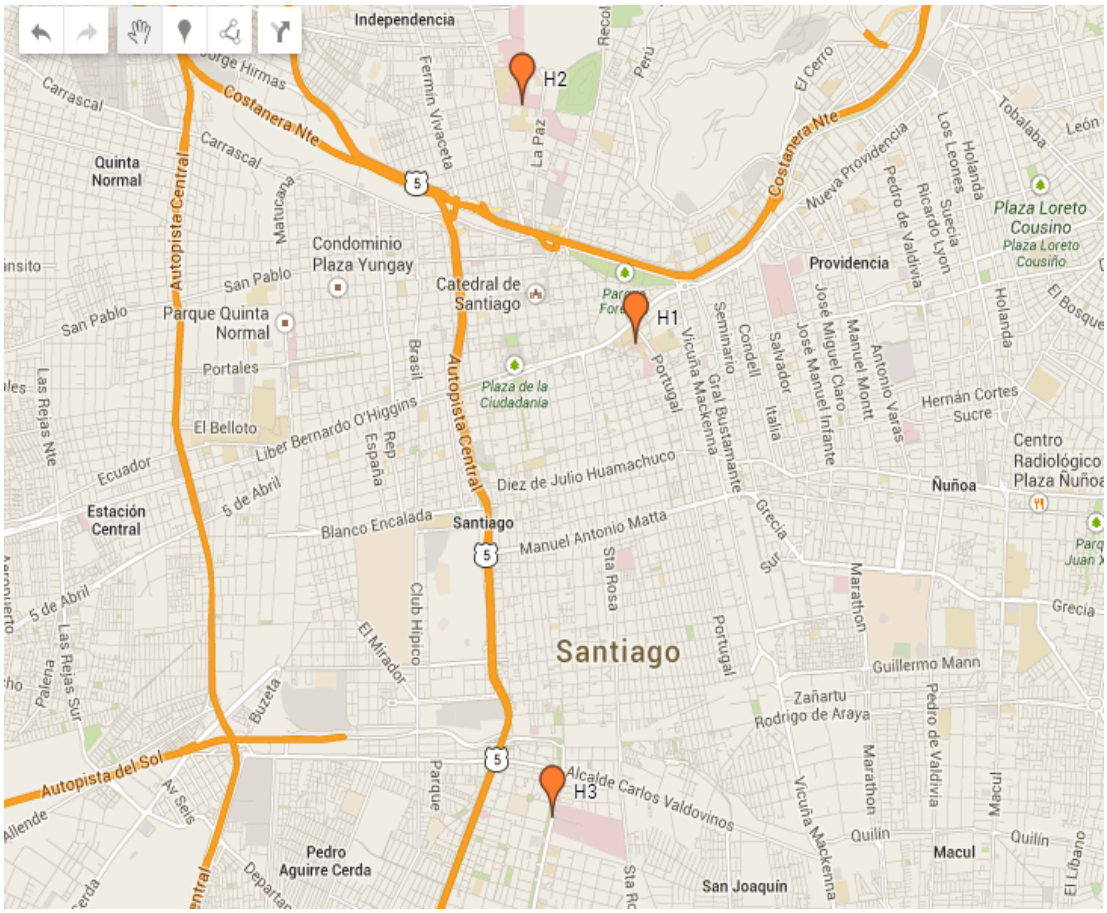


Figure 30. Geographic distribution of the hospitals from Santiago of Chile analysed. H1: Catholic University Hospital; H2: University of Chile Hospital; H3: Barros Luco Hospital.

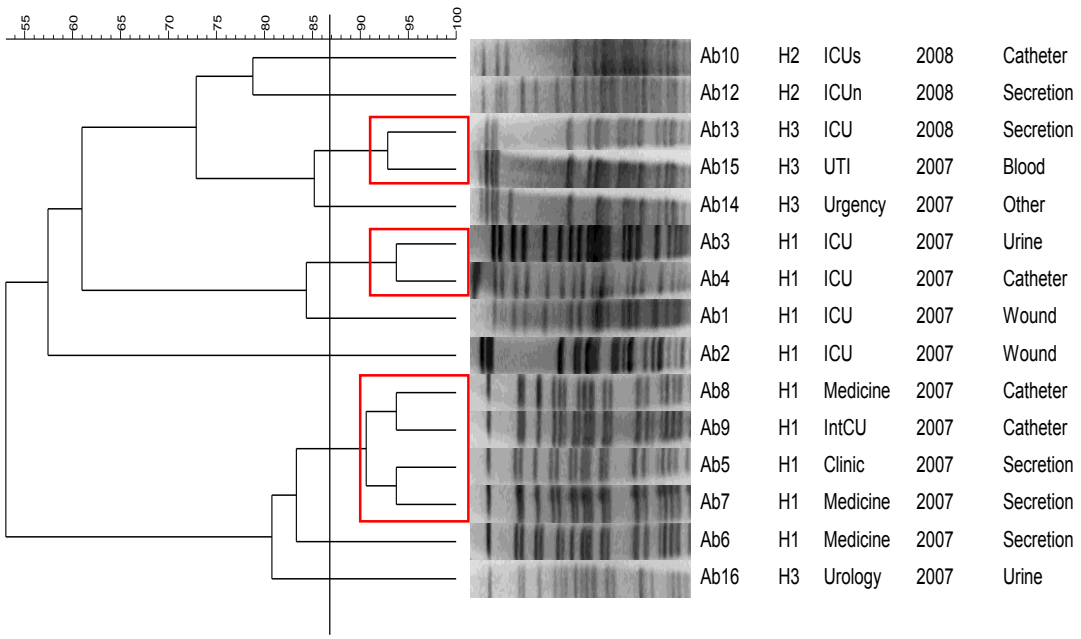


Figure 31. Dendogram showing the genetic diversity of *A. baumannii* from Chile determined by PFGE. The hospital, hospital wards, year and source of isolation are shown in the figure. H1: Catholic University Hospital; H2: University of Chile Hospital; H3: Barros Luco Hospital. ICU: Intensive care unit; IntCU: Intermediate care unit; ICUs: Intensive care unit (surgical); ICUs: Intensive care unit (neurology); ITU: Intensive therapy unit. The broken line corresponds to the cut-off level (≥87%) used to define the PFGE clonal clusters. The clonal groups are highlighted in red.

From the first hospital analysed (H1), two main clonal clusters were identified by PFGE (Figure 31). The first cluster comprises the Ab3 and Ab4 isolates from the same ward, whereas the second cluster includes the isolates Ab5, Ab7, Ab8 and Ab9, which were collected from three different hospital wards. The Ab1 isolate showed 85% similarity with the cluster formed by the Ab3 and Ab4 isolates, which indicates that the isolate Ab1 is related with them, maybe sharing a common origin. The Ab2 isolate showed a low similarity

with the other isolates obtained from this hospital. The presence of isolates that belong to the same clonal group in different wards may indicate that the strategy to control the dissemination of MDR clones, such as hand washing and surface disinfection, has failed in this hospital. It is now become increasingly important to perform a larger study of the prevalent clones in order to adjust the strategy needed to control the dissemination of these isolates within the hospital.

The isolates Ab10 and Ab12, collected from the University of Chile Hospital (H2); showed 79% similarity by PFGE (Figure 31). This indicates that they are probably not part of the same clonal group and they may have derived from different ancestors. This situation is similar to a study where 9 carbapenem-resistant *A. baumannii* isolates collected from the Aberdeen Royal Infirmary, Scotland, were analysed. The samples shared less than 80% similarity by PFGE and also harboured different $bla_{\text{OXA-51-like}}$ genes, such as $bla_{\text{OXA-51}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-65}}$, $bla_{\text{OXA-216}}$, $bla_{\text{OXA-217}}$ and $bla_{\text{OXA-89}}$ (Lopes *et al*., 2012a). These results showed that the clinical situation in this clinical centre was in a state of constant evolution, where new variants strains were continuously arising; this could be a similar situation to the one that is occurring in the Hospital H2.

Finally, the isolates Ab13 and Ab15 form part of the same clonal group from Barros Luco Hospital. The isolate Ab14 showed 85% similarity with Ab13 and Ab15, suggesting that they might share a common origin. Interestingly, they were isolated in different years from different hospital wards, which shows that these MDR-*A. baumannii* clones were transmitted and persistent within this hospital. Isolate Ab16 showed a low similarity with all the other isolates collected from the same hospital (H3) (Figure 31). However, it shows 80% similarity

with samples obtained from the Catholic University Hospital (H1), which may suggest that a common clone could be transferred between these hospitals.

In conclusion, these results show the presence of clonally related MDR-isolates within the hospital H1, which might suggest that the strategy to prevent the spread of these isolates is not effective. Additionally, it is important to emphasize that the Ab16 isolate, collected in the hospital H3, shared a high similarity (80%) with the clonal group identified in the hospital H1, which may be a consequence of the transfer of colonised patients between hospitals in Santiago. These findings highlight the need to implement a surveillance network including the most important hospitals from the country.

The isolates obtained from the UAE showed 92% of similarity by PFGE (Figure 32). The isolates shared less than 60% similarity with the first three classic European Clones, indicating that these isolates are part of different WW lineages.

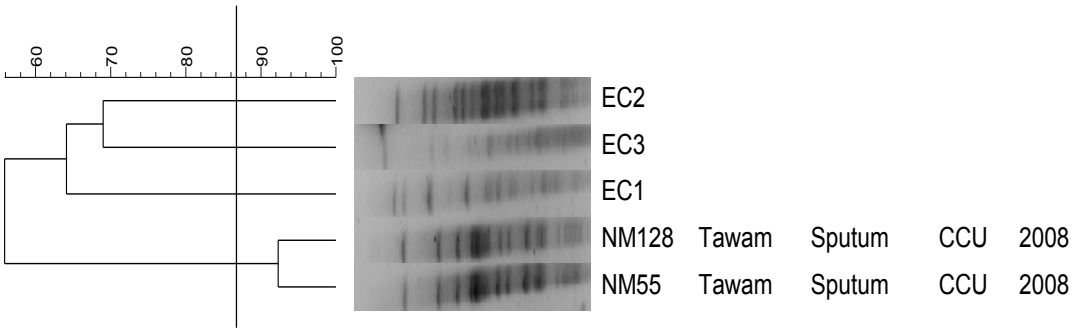


Figure 32. Dendogram showing the genetic diversity of *A. baumannii* the UAE determined by PFGE. CCU: Coronary care unit. The broken line corresponds to the cutoff level (≥87%) used to define the PFGE clonal clusters. EC1: European clone 1; EC2: European clone 2; EC3: European clone 3.

The isolates were considered isogenic as they were isolated consecutively from the same patient 4 months (first NM55 and NM128 later) apart and exhibited the similar PFGE patterns. These data suggest that these samples shared a common ancestor. Interestingly, despite the fact that they are isogenic, collected in the same year and ward, they have a different susceptibility pattern to ceftazidime. Specifically, NM55 was resistant to ceftazidime, whereas NM128 was susceptible to this drug. The reasons for this change are difficult to explain in an environment where cephalosporins, such as ceftazidime, are being used and it is an unusual example of spontaneous resistance loss in *A. baumannii*.

3.3. To detect and characterise the carbapenemases genes involved in the carbapenem-resistance in isolates from Chile and the UAE.

The intrinsic genes belonging to the *bla*$_{\text{OXA-51-like}}$ group had previously been detected as a method to identify the isolates as *Acinetobacter* spp. These results are shown in the section 3.1. However, is well known that individual members of the *bla*$_{\text{OXA-51-like}}$ group have a specific geographic distributions, related to epidemic lineages (Evans *et al*., 2008), thus making it necessary to know the sequence of the variants detected in Chile and in the UAE.

3.3.1. Chilean isolates

The members of the OXA-51-like group identified in the isolates from Chile were OXA-51, OXA-219, OXA-67 and OXA-64. A comparative analysis of the aminoacid sequences of these carbapenemases is shown in the Figure 33.

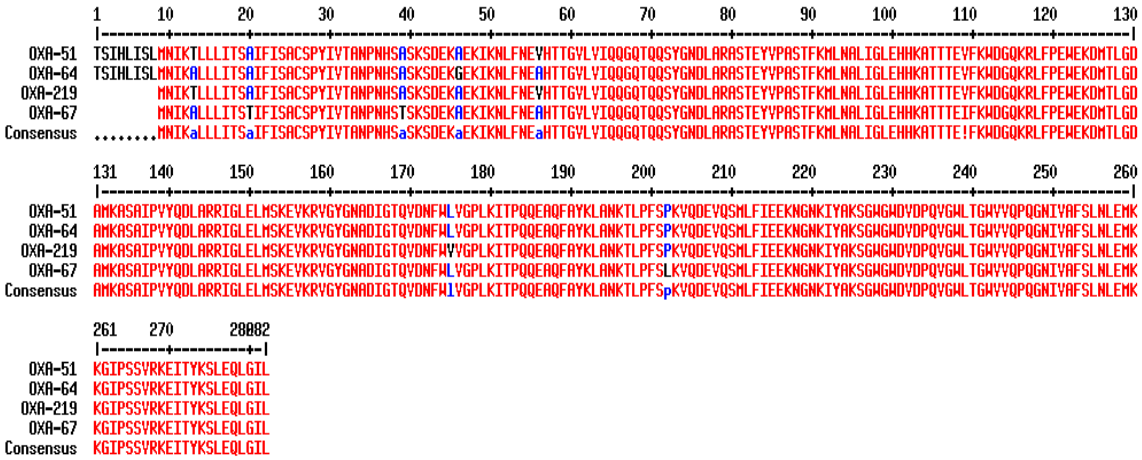


Figure 33. Multiple alignment of the OXA-51-like carbapenemases identified in the Chilean isolates. The changes in the sequence are indicated in blue and black.

The OXA-219 β-lactamase possesses one change in comparison with the parental enzyme OXA-51. This change is at position 175, where leucine was replaced by valine (Figure 33). Both aminoacids are hydrophobic, so the tertiary structure is not radically affected. In the case of the OXA-64 enzyme, there are three changes compared with OXA-51 (Figure 33). The changes are T13A, A46G and V56A. The substitution of threonine by alanine in the position 13 is significant as it is a replacement of a hydrophilic amino acid for a hydrophobic residue, which may affect the structure of the enzyme. Finally, the OXA-67 enzyme has four changes in its amino acid sequence. It has the same change as OXA-64 in the position 13, where threonine is substituted by alanine. Additionally, it has two substitutions, in the positions 20 and 29 (Figure 33), where a hydrophilic amino acid (alanine) was substituted by a hydrophobic residue (threonine). Finally, another significant change is at position 202, where proline was replaced by leucine, this is an important change in the secondary structure as proline can disrupt alpha helix and β-sheets structures (Morgan and Rubenstein, 2013).

The enzyme OXA-51 itself was identified in the isolates Ab5, Ab6, Ab7, Ab8 and Ab9, which were all collected in the hospital H1 (Figure 34). Additionally, this carbapenemase was also detected in the isolate Ab16 that was obtained in the hospital H3. The isolates Ab5, Ab7, Ab8 and Ab9 are part of the same clonal group (Figure 34), indicating that they may be derived from the same ancestor. Previously it had been shown by PFGE that isolate Ab6 showed 81% similarity with the samples described above, while the Ab16 had 80% similarity with them (Figure 31). In addition, they also carry the OXA-51 β-lactamase, confirming that they are related. As in the case of the clonal analysis, the sequencing results of the OXA-51 enzymes confirm that the Ab16 isolate may have been transferred from hospital H1 to hospital H3. It is important to note that this is the first report of the OXA-51 enzyme in *A. baumannii* from Chile.

The enzyme OXA-51 itself was originally detected in isolates from Argentina collected in 1996 that were resistant to carbapenems (Brown *et al*., 2005). Due to the close geographical relationship between Chile and Argentina, there is a high probability that some MDR-*A. baumannii* isolates could be transferred from one country to another, probably from Argentina to Chile as this enzyme was discovered much earlier in Buenos Aires (Brown *et al*., 2005).

Some isolates that belong to the same clonal group carry different OXA-51-like variants. Specifically the isolates Ab13 and Ab15 are part of the same cluster but they carry the OXA-67 and the OXA-64 enzymes, respectively. The same difference occurred in isolates Ab3 and Ab4, which were isolated from the hospital H1 and have 93% similarity by PFGE; however, they have different OXA-51-like variants, specifically OXA-219 and OXA-67, respectively (Figure 34). The determination of the clonal relatedness could be performed by different methods, such as PFGE or MLST; however, the sequencing of the $bla_{\text{OXA-51-like}}$ genes can represent a simpler and faster methodology. However, it has been shown that $bla_{\text{OXA-51-like}}$ gene sequencing correspond to MLST and sequence group typing, but not to PFGE (Zander *et al.*, 2012).

Additionally, as mentioned before, it is well known that some ICs or WWs are related with specific $bla_{\text{OXA-51-like}}$ variants (Evans *et al*., 2008). In this sense, a single-locus-based typing method, based in the sequence of $bla_{\text{OXA-51-like}}$ enzymes, has been proposed to assign *A. baumannii* isolates to ICs (Pournaras *et al*., 2014). Pournaras *et al.* conclude that this method classified accurately isolates of *A. baumannii* belonging to all major international clones; however, more studies are required to validate it as a reliable method for typing.

The OXA-51 itself has been identified in isolates from Chile and Brazil, which could not be related with any WW lineages, sharing just 77.9% similarity with the WW5 (Zander *et al*., 2012). Furthermore, the OXA-219 variant has been related to the WW4, which was previously detected in Chile, and also in Turkey, Argentina, India, Germany and Brazil (Zander *et al*., 2012). Moreover, the OXA-64 variants have been identified in the WW7, which in turn has been identified in Latvia, Switzerland, Venezuela, Mexico, Colombia, Singapore and Germany (Zander *et al*., 2012). Finally, the OXA-67 variant has been previously detected in isolates from Argentina (Brown and Amyes, 2005). However, in order to characterise them accurately as part of specific international clones, more experiments are required, as a multiplex-PCR amplifying 3 "housekeeping genes", *csuE*, *ompA* and $bla_{\text{OXA-51}}$, termed 3LST (Turton *et al*., 2007), which corresponds to a rapid and reliable typing method.

These results suggest that the MDR-isolates collected in Chile may belong to certain WW lineages that have been identified in other South American countries, such as in Argentina, Venezuela, Colombia and Brazil, suggesting the spread of these WW lineages among countries of the region. Nevertheless, more studies focused on the complete characterisation of the strains, such as the multiplex-PCR mentioned above, are necessary to conclude to which WW group they belong.

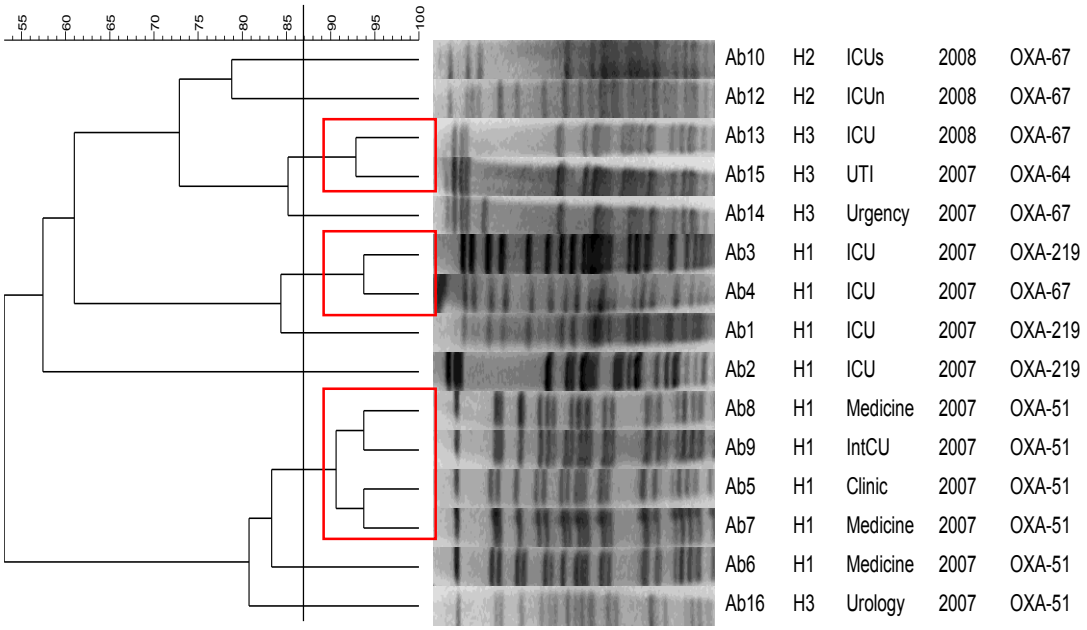


Figure 34. Dendogram showing the genetic diversity and the OXA-51-like variants of *A. baumannii* from Chile determined by PFGE. H1: Catholic University Hospital; H2: University of Chile Hospital; H3: Barros Luco Hospital. ICU: Intensive care unit; IntCU: Intermediate care unit; ICUs: Intensive care unit (surgical); ICUs: Intensive care unit (neurology); ITU: Intensive therapy unit. The broken line corresponds to the cutoff level (≥87%) used to define the PFGE clonal clusters. The clonal groups are highlighted in red.

3.3.2. Emirati isolates

Both Emirati isolates analysed carried the OXA-64 β-lactamase (Figure 35). As they are classified as isogenic strains, this was an expected result. According to Zander *et al*. (2012), this variant is related with the clonal lineage WW7, which has been detected in some European countries and in Latin America. Additionally, isolates containing this OXA-51-variant have been identified in South Africa (Brown and Amyes, 2005).

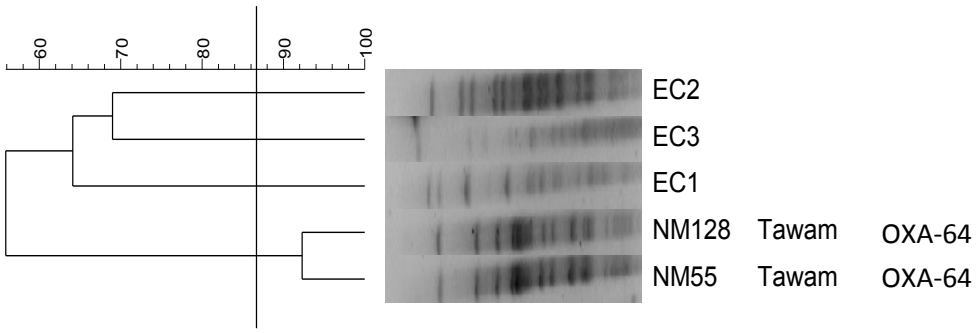


Figure 35. Dendogram showing the genetic diversity and the OXA-51-like variants of *A. baumannii* from the UAE determined by PFGE. The broken line corresponds to the cut-off level (≥87%) used to define the PFGE clonal clusters. EC1: European clone 1; EC2; European clone 2; EC3: European clone 3.

The samples from the UAE presented a low similarity with the main WW clones (ECs in the figure), and also they did not show similarities in the sequences of their $bla_{\text{OXA-51-like}}$ genes as the WW1 to -3 correlate with OXA-69, OXA-66 and OXA-71 β-lactamases respectively (Zander *et al*., 2012). Interestingly, this thesis is the first report of isolates containing the OXA-64 variant in Chile and in the UAE. According to the amino acid sequences, the OXA-64 enzyme is closely related with OXA-71, which is present in isolates that belong to the

WW3 clone (Evans *et al.*, 2008). Nevertheless, it is necessary to perform further analysis such as MLST in order to determine the WW (IC) to which the isolates belong.

3.3.3. Acquired carbapenem-hydrolysing class D β-lactamases (CHDLs)

It is well known that the resistance to carbapenems in *A. baumannii* is mediated mainly by the activity of OXA-type carbapenemases (Peleg *et al*., 2008). In this regard, the mere presence of the intrinsic OXA-51-like enzymes does not contribute towards the carbapenem-resistance. Their specific contribution depends on its association with insertion sequences, such as IS*Aba1* upstream the $bla_{\text{OXA-51-like}}$ genes (Turton *et al*., 2006a), which provides a strong promoter. The presence of this association is investigated in the next aim of this thesis. On the other hand, the acquired CHDLs play a key role in the resistance to carbapenems in *A. baumannii.* Due to the above, the presence of acquired CHDLs, such as OXA-type carbapenemases, was carried out by PCR of their genes.

The acquired CHDL present in the Chilean isolates were screened by the multiplex PCR proposed by Woodford *et al.* (2006). This multiplex PCR includes the OXA-51-like, OXA-23-like, OXA-24-like and the OXA-58-like carbapenemases. An example gel of this PCR is shown in the Figure 36. The sizes of the products for each group are 353bp for $bla_{\text{OXA-51-like}}$, 501bp for $bla_{\text{OXA-23-like}}$, 246bp for $bla_{\text{OXA-24-like}}$ and 599 for $bla_{\text{OXA-58-like}}$. As two new groups of carbapenemases, OXA-143 and OXA-235, were described after the multiplex-PCR proposed by Woodford *et al*. (2006), they were screened by separate standard PCR using the primers and conditions proposed by Lopes (PhD Thesis in Biomedical Sciences, The University of Edinburgh, 2012) and Higgins *et al*. (2013).

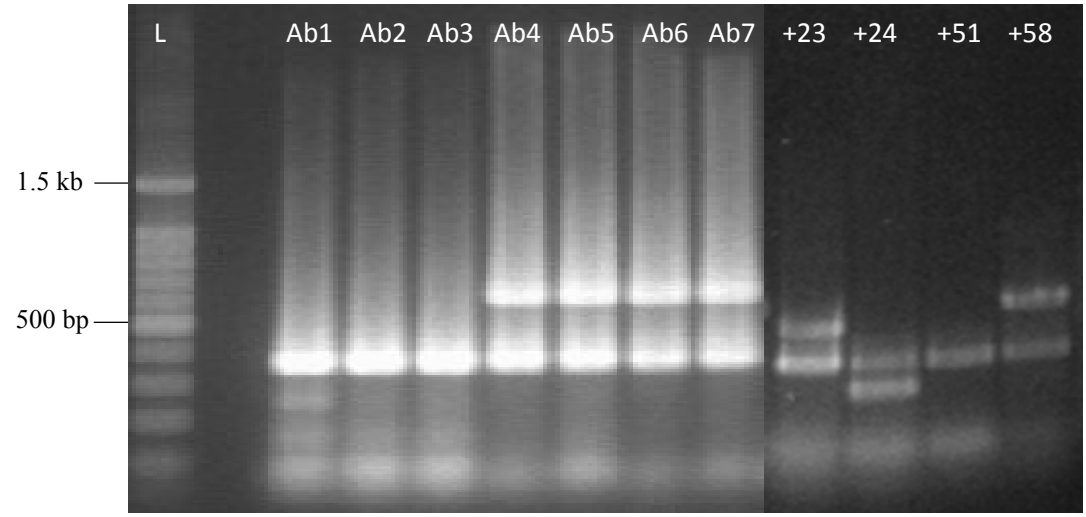


Figure 36. Example gel of amplification of the acquired CHDLs by multiplex PCR. L: 100bp DNA ladder; +23: *bla*$_{\text{OXA-23-like}}$ positive control; +24: *bla*$_{\text{OXA-24-like}}$ positive control; +51: *bla*$_{\text{OXA-51-like}}$ positive control; +58: *bla*$_{\text{OXA-58-like}}$ positive control.

The results of the multiplex PCR and the MICs of carbapenems are summarised in Table 8. The most prevalent acquired CHDL in the samples from Chile was the OXA-58 carbapenemase, which was present in 12 of the 15 samples analysed (Table 8). In the case of the Emirati isolates, the presence of OXA-type carbapenemases was previously investigated in the laboratory of Professor Tibor Pál, in the UAE. Both samples, NM55 and NM128, were positive for the *bla*$_{\text{OXA-23-like}}$ as an acquired CHDL. These results were confirmed by myself using the specific primers for *bla*$_{\text{OXA-23-like}}$ genes proposed by Woodford *et al*. (2006) (Figure 37). Additionally, Professor Tibor Pál supplied the MICs values of the carbapenems (Table 8).

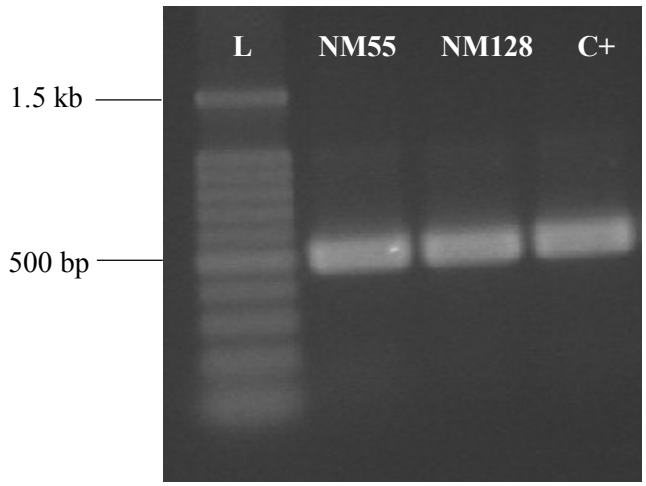
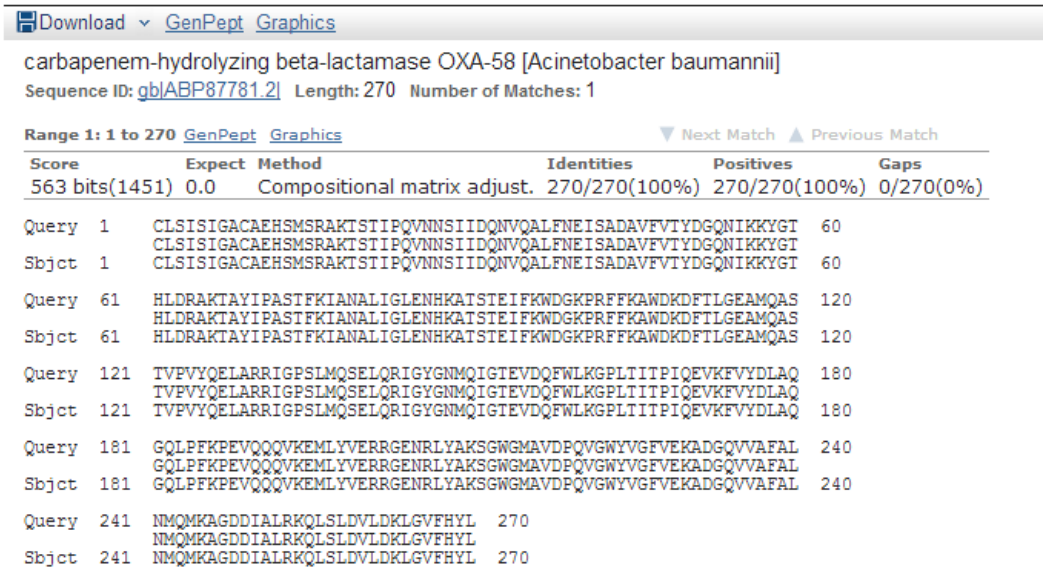


Figure 37. Confirmation of the presence of the *bla*$_{\text{OXA-23-like}}$ gene by PCR. C+: positive control *A. baumannii* previously determined in our laboratory as *bla*$_{\text{OXA-23-like}}$ positive; L: 100bp DNA ladder.

Table 8. OXA-type carbapenemases present in the Chilean isolates. H1: Catholic University Hospital; H2: University of Chile Hospital; H3: Barros Luco Hospital. CHDL: Carbapenem hydrolysing class D β-lactamase.

Strain	**Hospital**	***bla*$_{\text{OXA-51-like}}$**	**Acquired CHDL**	**IMP MIC (mg/l)**	**MEM MIC (mg/l)**
Ab1	H1	OXA-219	-	16	32
Ab2	H1	OXA-219	-	16	32
Ab3	H1	OXA-219	-	16	32
Ab4	H1	OXA-67	OXA-58	16	8
Ab5	H1	OXA-51	OXA-58	16	8
Ab6	H1	OXA-51	OXA-58	16	8
Ab7	H1	OXA-51	OXA-58	16	8
Ab8	H1	OXA-51	OXA-58	16	8
Ab9	H1	OXA-51	OXA-58	8	4
Ab10	H2	OXA-67	OXA-58	32	8
Ab12	H2	OXA-67	OXA-58	32	8
Ab13	H3	OXA-67	OXA-58	32	8
Ab14	H3	OXA-67	OXA-58	32	8
Ab15	H3	OXA-64	-	8	16
Ab16	H3	OXA-51	OXA-58	32	8
NM55	Tawam	OXA-64	OXA-23	>32	>32
NM128	Tawam	OXA-64	OXA-23	32	24

The *bla*$_{\text{OXA-58-like}}$ genes have been previously identified in carbapenem-resistant isolates from South America, specifically in Argentina, Colombia, Bolivia and Venezuela (Higgins *et al.*, 2010; Lopes *et al*., 2013; Merkier *et al*., 2008; Opazo *et al*., 2012b). Even though the most frequent OXA-type carbapenemases detected in South America are OXA-23-like and OXA-24-like, during 2007-2008 the case in Chile was different, as isolates containing enzymes of the OXA-58 group were more prevalent (Gales *et al*., 2012; Opazo *et al*., 2012b). The sequencing of the PCR products showed the presence of the OXA-58 enzyme itself, in the Chilean isolates (Figure 38).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carbapenem-hydrolyzing beta-lactamase OXA-58 [Acinetobacter baumannii]

Sequence ID: gb|ABP87781.2| Length: 270 Number of Matches: 1

Range 1: 1 to 270 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
563 bits(1451)	0.0	Compositional matrix adjust.	270/270(100%)	270/270(100%)	0/270(0%)

```
Query  1    CLSISIGACAEHSMSRAKTSTIPQVNNSIIDQNVQALFNEISADAVFVTYDGQNIKKYGI  60
            CLSISIGACAEHSMSRAKTSTIPQVNNSIIDQNVQALFNEISADAVFVTYDGQNIKKYGI
Sbjct  1    CLSISIGACAEHSMSRAKTSTIPQVNNSIIDQNVQALFNEISADAVFVTYDGQNIKKYGI  60

Query  61   HLDRAKTAYIPASTFKIANALIGLENHKATSTEIFKWDGKPRFFKAWDKDFTLGEAMQAS  120
            HLDRAKTAYIPASTFKIANALIGLENHKATSTEIFKWDGKPRFFKAWDKDFTLGEAMQAS
Sbjct  61   HLDRAKTAYIPASTFKIANALIGLENHKATSTEIFKWDGKPRFFKAWDKDFTLGEAMQAS  120

Query  121  TVPVYQELARRIGPSLMQSELQRIGYGNMQIGTEVDQFWLKGPLTITPIQEVKFVYDLAQ  180
            TVPVYQELARRIGPSLMQSELQRIGYGNMQIGTEVDQFWLKGPLTITPIQEVKFVYDLAQ
Sbjct  121  TVPVYQELARRIGPSLMQSELQRIGYGNMQIGTEVDQFWLKGPLTITPIQEVKFVYDLAQ  180

Query  181  GQLPFKPEVQQQVKEMLYVERRGENRLYAKSGWGMAVDPQVGWYVGFVEKADGQVVAFAL  240
            GQLPFKPEVQQQVKEMLYVERRGENRLYAKSGWGMAVDPQVGWYVGFVEKADGQVVAFAL
Sbjct  181  GQLPFKPEVQQQVKEMLYVERRGENRLYAKSGWGMAVDPQVGWYVGFVEKADGQVVAFAL  240

Query  241  NMQMKAGDDIALRKQLSLDVLDKLGVFHYL  270
            NMQMKAGDDIALRKQLSLDVLDKLGVFHYL
Sbjct  241  NMQMKAGDDIALRKQLSLDVLDKLGVFHYL  270
```

Figure 38. Comparison of the *bla*$_{\text{OXA-58-like}}$ gene of the Ab6 isolate with the sequence available in the NCBI database (http://www.ncbi.nlm.nih.gov/). Query: Nucleotide sequence of *bla*$_{\text{OXA-58-like}}$ of isolate Ab6; Sbjct: Subject sequence in the NCBI database.

As shown in the Table 8, the MICs of carbapenems of the Emirati isolates that contained the acquired OXA-23-like carbapenemase are higher than the MICs of the Chilean isolates that

harbour the OXA-58 enzyme. When the values of the MICs are compared between isolates that contain the OXA-64 variant as a common intrinsic enzyme (isolates Ab15, NM55 and NM128), the presence of the OXA-23-like carbapenemase is crucial for provide higher resistance to carbapenems, suggesting that the impact of OXA-23-like β-lactamase to the resistance to carbapenems is greater than the effect of OXA-58. In this sense, according to a study where the hydrolytic activities of different acquired OXA-type carbapenemases were analysed, it was determined that OXA-58 has the lowest activity in comparison with OXA-24 and OXA-23 (Héritier *et al.*, 2005b). Interestingly, when the $bla_{\text{OXA-58}}$ gene was cloned into a commercial plasmid and then incorporated into a carbapenem-susceptible *A. baumannii* strain, the MICs of carbapenems were lower than when the natural plasmid harbouring the $bla_{\text{OXA-58}}$ gene was incorporated into the same susceptible strain. Specifically, when the susceptible strain CIP70.10 was transformed with the natural plasmid containing the OXA-58 β-lactamase, the MICs of imipenem and meropenem were each 16mg/l. When the same strain was transformed using commercial plasmids that contain the same carbapenemase gene, the MICs of imipenem and meropenem were 0.5mg/l. These differences may be due to the presence of IS-elements, such as IS*Aba3*, associated with the carbapenemase gene in the natural occurring plasmid, which could increase the expression of the gene, leading to the resistance to these compounds (Héritier *et al*., 2005b).

The $bla_{\text{OXA-58-like}}$ gene was the most prevalent in the isolates studied in this thesis, which were collected during 2007-2008; however, in a study where 90 carbapenem-resistant *A. baumannii* collected between 2011 and 2012 from Chile were analysed, the most prevalent acquired OXA-type carbapenemase was OXA-23-like (Opazo Alexis A., Personal Communication, 2014). These differences represent an important change in the mechanisms of resistance to carbapenems, as OXA-23 has a higher hydrolytic activity than OXA-58, and therefore the MICs of carbapenems are higher. The appearance of OXA-23 in *A. baumannii*

may be the result of the mobilisation of resistant strains from neighbouring countries where these enzymes have been previously detected.

According to the Table 8, the MICs values of meropenem of the isolates Ab1, Ab2 and Ab3, are the highest among the Chilean samples, in that they do not contain any known acquired OXA-type carbapenemase; however, they harboured the OXA-219 enzyme as a member of the intrinsic OXA-51-like enzymes. According to Zander *et al.* (2012), some carbapenem-resistant isolates, which harboured the OXA-219 enzyme, were also positive for the presence of IS*Aba1* upstream the $bla_{\text{OXA-219}}$ gene and did not possess an extra CHDL. Interestingly, the isolate Ab15 did not show any acquired CHDL and harboured the OXA-64 as member of the intrinsic OXA-51-like group (Table 8). Unlike the isolates Ab1, Ab2 and Ab3, which did not contain any extra CHDL and possessed the OXA-219 variant, the MIC of imipenem was comparatively lower; being intermediate, according to the BSAC guidelines (Andrews, 2010), whereas its MIC of meropenem was higher and classified it as meropenem-resistant (Table 8). These differences may be due the presence of an extra non-enzymatic mechanism of resistance, such as loss of porins and active efflux (Peleg *et al*., 2008), which may be affecting the activity of meropenem.

In the case of the Emirati isolates, the $bla_{\text{OXA-23}}$ gene has been previously detected in countries from the Arabian Peninsula. Specifically, *A. baumannii* isolates carrying the OXA-23 enzyme have been identified in Bahrain, the UAE and Saudi Arabia (Mugnier *et al*., 2010; Zowawi *et al*., 2013). In the case of the isolate from Bahrain, it was collected in 2008 and the genetic platform associated with the $bla_{\text{OXA-23}}$ comprised the IS*Aba1* element which, in turn, was identified on a plasmid of ca.130kb (Mugnier *et al*., 2010). Interestingly, the $bla_{\text{OXA-23}}$ embedded in the same genetic platform was detected in an isolate from the UAE

that was collected two years before the isolation of the Bahraini sample (Mugnier *et al*., 2010). Additionally, another isolate that harboured the *bla*$_{\text{OXA-23}}$ gene, collected in 2006 from the UAE, presented a different genetic platform that comprised Tn*2006* and was embedded in the chromosome (Mugnier *et al*., 2010).

In the case of Saudi Arabia, the analysis of 56 isolates obtained from Riyadh, collected between 2010 and 2011, showed that the 53.6% harboured the *bla*$_{\text{OXA-23}}$ carbapenemase (reviewed by Zowawi *et al*., 2013). Furthermore, of 132 *Acinetobacter* isolates from the eastern province of Saudi Arabia, 79.5% of the samples carried the *bla*$_{\text{OXA-23}}$ gene (reviewed by Zowawi *et al*., 2013). The results shown above represent a novel detection of the *bla*$_{\text{OXA-23}}$ gene in the Arabian Peninsula, specifically in the UAE (Opazo *et al*., 2012a). The association of this gene with any insertion sequence is analysed in the next aim of this thesis. According to Zowawi *et al*. (2013), the OXA-23 enzyme is the most prevalent in the Arabian Peninsula, representing one of the most important mechanisms of carbapenem-resistance in *A. baumannii* from this part of the world.

3.4. To identify mobile genetic elements mediating the carbapenem-resistance in isolates from Chile.

3.4.1. Association between IS*Aba1* and *bla*$_{\text{OXA-51-like}}$.

This section analysed the association of the resistance genes detected with mobile genetic elements, such as insertion sequences, plasmids, etc. As shown previously, the isolates Ab1, Ab2, Ab3 and Ab15 did not contained a CHDL in addition to the OXA-51-like enzyme, suggesting that the OXA-51-like β-lactamase may explain their resistance to carbapenems. It is known that the presence of IS*Aba1* can regulate the expression of OXA-type carbapenemases, such as OXA-51-like (Turton *et al*., 2006a), therefore, PCR experiments detecting the presence of IS*Aba1* upstream the *bla*$_{\text{OXA-51-like}}$ gene were performed in the samples that did not contained an extra CHDL.

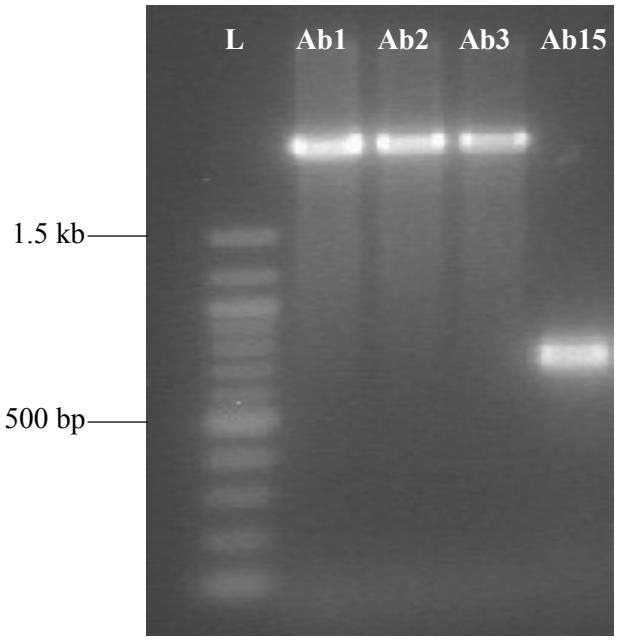


Figure 39. Detection of IS*Aba1* upstream the *bla*$_{\text{OXA-51-like}}$ gene. L: 100bp DNA ladder.

As shown in the Figure 39, the isolates Ab1, Ab2 and Ab3 produced an amplicon larger than 1.5kb, which indicates the presence of an IS element upstream the *bla*$_{\text{OXA-51-like}}$ gene. The

sequencing of these products confirmed the presence of IS*Aba1* associated upstream the carbapenemase gene. The presence of IS*Aba1* in the isolates described above should explain their increased resistance profiles to carbapenems, in the absence of a transferable CHDL (Table 8). It is important to note that the role of the $bla_{\text{OXA-51-like}}$ in carbapenem resistance in *A. baumannii* depends on the presence of IS*Aba1* (Turton *et al*., 2006a); therefore, two main mechanisms of carbapenem-resistance, such as the presence of OXA-58 and association of IS*Aba1* with $bla_{\text{OXA-51-like}}$, have been detected in this thesis, as OXA-23, OXA-24, OXA-143 and OXA-235 β-lactamases were not detected in the isolates from Chile.

It is interesting to note the versatility of the IS*Aba1* element as it has been identified upstream of the $phaB_{\text{AC}}$ gene, which encodes an acetoacetyl-CoA reductase, and has also been detected upstream the $bla_{\text{OXA-23-like}}$ gene in *A. baumannii* (reviewed by Evans *et al*., 2013). Additionally, it can be present upstream of the $bla_{\text{ADC-like}}$ genes, which leads to the resistance to cephalosporins (Héritier *et al*., 2006). Moreover, it has been detected disrupting the *carO* gene, thus preventing its expression and preventing the penetration of the antibiotics from the external media (Lee *et al*., 2011). Furthermore, IS*Aba1* has been identified regulating the expression of *sulII* (Segal *et al*., 2005), *aac6'Ib* (Naas *et al*., 2007), *aacC2* (Segal *et al*., 2003) and $bla_{\text{OXA-58-like}}$ (Poirel and Nordmann, 2006); hence, this genetic element has an important role in the resistance to different types of antibiotics, such as carbapenems, cephalosporins, sulphonamides and aminoglycosides, among others. In the specific case of the $bla_{\text{OXA-51-like}}$ genes, it has been found that the IS*Aba1* produces an increment of the expression of this gene. In a work published in 2009, it was demonstrated that when the IS*Aba1* element was inserted, *in vitro*, upstream the $bla_{\text{OXA-51-like}}$ gene, the MICs values doubled to imipenem (from 2 to 4mg/L) and to meropenem (from 3 to 6mg/L) (Figueiredo *et al*., 2009a). Interestingly, multiple copies of this genetic element are present in the genome of *A. baumannii* (Segal *et al*., 2005), which could represent a natural arsenal of

different mechanisms of antibiotic resistance, giving all *Acinetobacter baumannii* the potential to become MDR when they are under antibiotic pressure. Additionally, the presence of multiple copies of this IS element may reflect its mobility and could suggest that transposition events occur frequently, producing a variety of arrangement in the genetic material of *A. baumannii* (Segal *et al*., 2005).

As mentioned earlier in this thesis, the *bla*$_{\text{OXA-23-like}}$ gene has been identified lately in *A. baumannii* collected in Chile between 2011 and 2012, which showed the presence of the IS*Aba1* upstream this OXA-type carbapenemase gene (Opazo Alexis A, Personal communication, 2014). This finding reflects the importance of the activity of IS*Aba1* in the regulation of carbapenem-resistance by modulating genes that have been recently identified in the country.

The association between IS*Aba1* and *bla*$_{\text{OXA-51-like}}$ in *A. baumannii* is not new in South America. In a study published in 2012, where 20 isolates from Colombia were analysed, four isolates carried the IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene and additionally six samples contained the IS*Aba1* upstream the *bla*$_{\text{OXA-23-like}}$ (Martínez and Mattar, 2012). In this work, the IS*Aba1* was also mediating the cephalosporin-resistance as it was detected upstream the *bla*$_{\text{ADC-7}}$ gene. Interestingly, in a study where carbapenem-resistant isolates from Bolivia were analysed, none of them had insertion sequences upstream the *bla*$_{\text{OXA-51-like}}$ gene (Lopes *et al*., 2013). Additionally, isolates from Chile containing the IS*Aba1* upstream the *bla*$_{\text{OXA-219}}$ gene, have been detected (Zander *et al*., 2012). These findings reflect the major importance of IS*Aba1* in the modulation of the antibiotic resistance not only to carbapenems, but also reflects that the variability of mechanisms of resistance that can be present in this pathogen.

3.4.2. Genetic environment of *bla*$_{OXA-58}$ in the isolates from Chile

As the *bla*$_{OXA-58}$ gene could be present in both plasmids and chromosome (Opazo *et al*., 2012b), it is important to analyse in which replicon this gene is located. The plasmids present in the Chilean isolates were extracted using a commercial kit described in the Chapter 2 and then separated by PFGE. An example gel of the plasmid profiles is shown in the Figure 40. As shown in this figure, the strains possessed different plasmids of varying sizes. As the isolates Ab1, Ab2, Ab3 and Ab15 did not harbour the *bla*$_{OXA-58}$ gene, they were excluded from the plasmid extraction experiments.

The isolates harboured large plasmids, most of them larger than 100kb (Figure 40). This fact reflects the ability of *A. baumannii* to capture exogenous DNA, which can provide diverse functions, such as antibiotic resistance.

Probably, these plasmids encode not only antibiotic resistance genes, but also could encode metabolic genes, metal resistance genes, transposition genes, among others. In order to characterise the pool of plasmids present in the isolates, it is necessary to perform further analysis, such as the determination of the incompatibility group for each plasmid (Bertini *et al*., 2010) and analyse which is the most prevalent in these isolates.

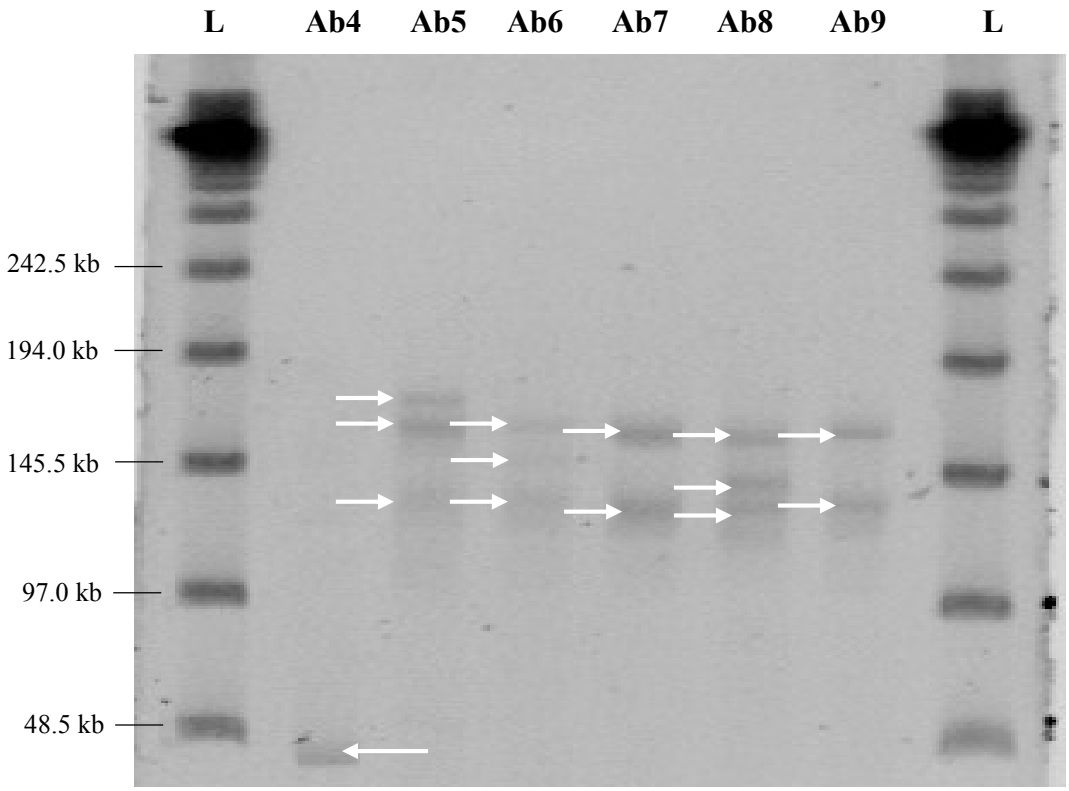


Figure 40. Example gel of plasmid separated by PFGE. L: Lambda ladder PFG marker. Each white arrow indicates a plasmid present in the isolates.

After the plasmid extraction and separation by PFGE, the bands were extracted from the agarose gel and the DNA was used as template for PCR experiments (Figure 41A). In order to ensure that the DNA extracted corresponded exclusively to plasmid DNA, a PCR to detect the chromosomal 16S gene was performed (Figure 41B).

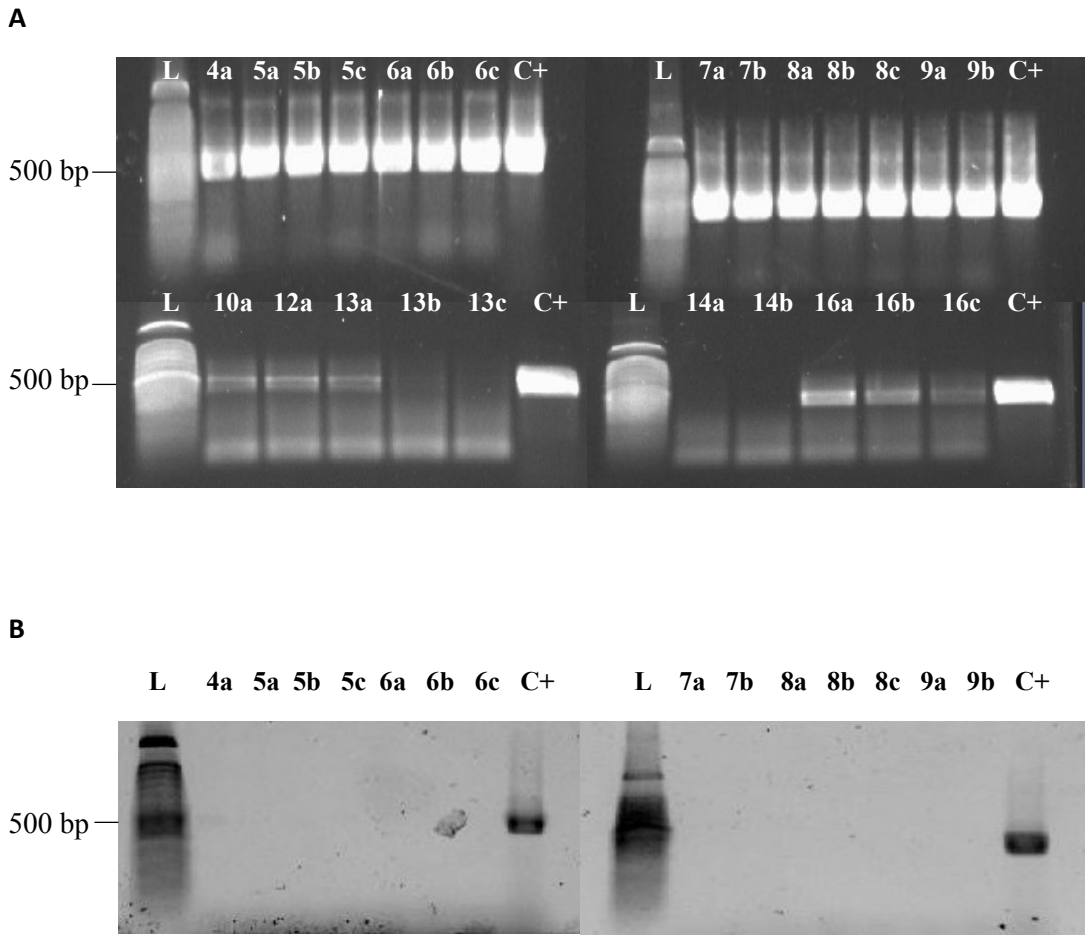


Figure 41. Detection of *bla*$_{OXA-58}$ in plasmids from Chilean isolates. A: Detection of *bla*$_{OXA-58}$ using the plasmid DNA as template. The letters after the isolate number represent the different plasmids detected in each isolate. B: Example gel of the detection of the chromosomal *16S* gene in the plasmid DNA. C+: Total DNA from Ab1 used as positive control. L: 100bp DNA ladder.

Of all the Chilean samples analysed, ten isolates harboured the *bla*$_{OXA-58}$ gene in plasmids (Figure 41). Interestingly, the *bla*$_{OXA-58}$ gene was detected in more than one plasmid in each sample, indicating that most of them have multiple copies of this gene, as shown in Figure 41. The exception was isolate Ab13, which had three plasmids (ca.50kb, ca.150kb and ca.240 kb) and the *bla*$_{OXA-58}$ gene was present only in the largest plasmid (Figure 41).

Only the samples from Ab4 and Ab10 presented a single plasmid that, in both cases, harboured the carbapenemase gene (Figure 41). The isolate Ab14 showed two plasmids (ca.48kb and ca.150kb), neither of which contained the *bla*$_{\text{OXA-58}}$ gene, suggesting that this gene was carried in the chromosome (Figure 41).

These results confirm the trend in South America that the location of the *bla*$_{\text{OXA-58}}$ gene is mainly on plasmids. In a study, where 46 isolates collected during 2008 and 2009 in Bolivia were analysed, the *bla*$_{\text{OXA-58}}$ gene was detected in plasmids of ca.40kb (Sevillano *et al*., 2012). In another work carried out in Argentina in 2011, the *bla*$_{\text{OXA-58}}$ was detected on plasmids, in which the IS*Aba825* element was associated upstream the carbapenemase gene (Ravasi *et al*., 2011). These findings suggest that the potential dissemination of this acquired CHDL is high, as it is mainly harboured on mobile genetic elements, which could be mobilised intra- or inter-species.

The association of *bla*$_{\text{OXA-58}}$ with insertion sequences was analysed by PCR as described earlier in the Materials and Methods chapter. The primers described in the Chapter 2 allow the detection of any IS sequence upstream the *bla*$_{\text{OXA-58}}$ gene; therefore, after the amplification of this region by PCR, it was necessary to sequence the products. An example gel of the PCR amplification is shown in the Figure 42.

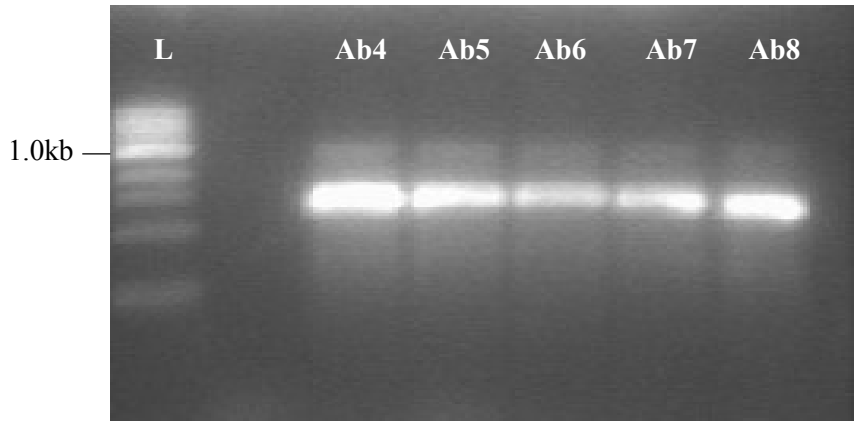


Figure 42. Example gel of the detection of IS*Aba3* upstream the *bla*$_{\text{OXA-58}}$ gene in isolates from Chile. L: 1kb DNA ladder.

Of the eleven samples analysed, the Ab5, Ab6, Ab7, Ab8, Ab9 and Ab16 showed the presence of an IS element upstream the *bla*$_{\text{OXA-58}}$ gene (Figure 42). The PCR products were sequenced and compared with the NCBI database (http://www.ncbi.nlm.nih.gov/), revealing that all the positive isolates harboured the IS*Aba3* element both upstream and downstream (Figure 43). In addition, the sequences were compared with the data available in ISFinder (https://www-is.biotoul.fr/, last accessed February 17th, 2014) as it exclusively contains sequences of IS elements, confirming the presence of IS*Aba3* both upstream and downstream.

GGTGGTGCTTCAAAAAGTATGCTGCCTTTAAATGAAGCCATTATTGATGTAAAAGAAAAGAAGGCTAAATCAAAAG
CTTTAAAATGGTTTTCAAGCTTTTTCGAAAAATTACAGCTTCTTCTGAAGCTACGCCTAATTCTATGCCTTATTTT
GCAATTATTGCCTTCAATGCCTACAGTAAAAAATTTACCAATACTTTGCTTACCATTTTTAAAAGCAGTGATGAAA
CTGTCCCAATGATCACTTGCAATTCGGGTGTAGTGAATATCTAATTGTTTAAGCTTTGTCTTCAATTGTTAGACAG
TAGCTAAATCTCGTTTACCCCAAACATAAGCAACAATTTCACCTGTTTCTCGATGATAGGCGTAAATAAGCCATTG
TTTATTCTTTTTATTTCCAACAAAATTCCAGAACTCATCTACTTCGAGAGATTCATAATGACTTTGCTGAGGCTGA
ATTTCATAGGTTGATTCAGTTAAAGTACGTAAAACTTTACCGATACTGATGCGCTCAACTTCAGCAATATCTCGTA
TACCGCTGCCTCTGACCATCAACTGTAATATTTTACGAGTAATACCTGACTTACATCCTAGATAGCTCAGTGCATG
ATCACCAATAAACTGACGTTTACAGTCTTTGCACTGATAGTTTTGTTTCCCATCTACTTTGATACCATTTTTCTTT
ATACTATCACTGAGGCAGGTTGGACATTTGATTGCTAGAGTTATTTGCATTTCTCTATTTTATCAAAATCCAATCG
GCTTTTTCTTCAGCATACTTTTTGAAACACTACCTTTTAAAGTTGTATATCTGCTTAAGCATAAGTATTGGGGCTT
GTGCTGAGCATAGTATGAGTCGAGCAAAAACAAGTACAATTCCACAAGTGAATAACTCAATCATCGATCAGAATGT
TCAAGCGCTTTTTAATGAAATCTCAGCTGATGCTGTGTTTGTCACATATGATGGTCAAAATATTAAAAAATATGGC
ACGCATTTAGACCGAGCAAAAACAGCTTATATTCCTGCATCTACATTTAAAATTGCCAATGCACTAATTGGTTTAG
AAAATCATAAAGCAACATCTACAGAAATATTTAAGTGGGATGGAAAGCCACGTTTTTTTAAAGCATGGGACAAAGA
TTTTACTTTGGGCGAAGCCATGCAAGCATCTACAGTGCCTGTATATCAAGAATTGGCACGTCGTATTGGTCCAAGC
TTAATGCAAAGTGAATTGCAACGTATTGGTTATGGCAATATGCAAATAGGCACGGAAGTTGATCAATTTTGGTTGA
AAGGGCCTTTGACAATTACACCTATACAAGAAGTAAAGTTTGTGTATGATTTAGCCCAAGGGCAATTGCCTTTTAA
ACCTGAAGTTCAGCAACAAGTGAAAGAGATGTTGTATGTAGAGCGCAGAGGGGAGAATCGTCTATATGCTAAAAGT
GGCTGGGGAATGGCTGTAGACCCGCAAGTGGGTTGGTATGTGGGTTTTGTTGAAAAGGCAGATGGGCAAGTGGTGG
CATTTGCTTTAAATATGCAAATGAAAGCTGGTGATGATATTGCTCTACGTAAACAATTGTCTTTAGATGTGCTAGA
TAAGTTGGGTGTTTTTCATTATTTATAATTTTAAAGTTGTATATCGGTAGTGTTTCAAAAAGTATGCTGAAGAAAA
AGCCGATTGGATTTTGATAAAATAGAGAAATGCAAATAACTCTAGCAATCAAATGTCCAACCTGCCTCAGTGATAG
TATAAAGAAAAATGGTATCAAAGTAGATGGGAAACAAAACTATCAGTGCAAAGACTGTAAACGTCAGTTTATTGGT
GATCATGCACTGAGCTATCTAGGATGTAAGTCAGGTATTACTCGTAAAATATTACAGTTGATGGTCAGAGGCAGCG
GTATACGAGATATTGCTGAAGTTGAGCGCATCAGTATCGGTAAAGTTTTACGTACTTTAACTGAATCAACCTATGA
AATTCAGCCTCAGCAAAGTCATTATGAATCTCTCGAAGTAGATGAGTTCTGGAATTTTGTTGGAAATAAAAAGAAT
AAACAATGGCTTATTTACGCCTATCATCGAGAAACAGGTGAAATTGTTGCTTATGTTTGGGGTAAACGAGATTTAG
CTACTGTCTAACAATTGAAGACAAAGCTTAAACAATTAGATATTCACTACACCCGAATTGCAAGTGATCATTGGGA
CAGTTTCATCACTGCTTTTAAAAATGGTAAGCAAAGTATTGGTAAATTTTTTACTGTAGGCATTGAAGGCAATAAT
TGCAAAATAAGGCATAGAATTAGGCGTAGCTTCAGAAGAAGCTGTAATTTTTCGAAAAAGCTTGAAAACCATTTTA
AAGCTTTTGATTTAGCCTTCTTTTCTTTTACATCAATAATGGCTTCATTTAAAGGCAGCATACTTTTTGAAGCACC
ACC

Figure 43. Nucleotide sequence of the genetic environment of the *bla*$_{\text{OXA-58}}$ present in isolate Ab5. Red underlined nucleotides: intergenic regions. Blue underlined nucleotides IS*Aba3*-elements. Not underlined nucleotides: *bla*$_{\text{OXA-58}}$ gene.

In the six *bla*$_{\text{OXA-58}}$-positive isolates from Chile, this gene was embedded in a transposon-like structure, flanked by two copies of IS*Aba3* one upstream and the other downstream (Figure 44). There was a spacer region of 17bp present between the *bla*$_{\text{OXA-58}}$ gene and the IS*Aba3*-elements both upstream and downstream.

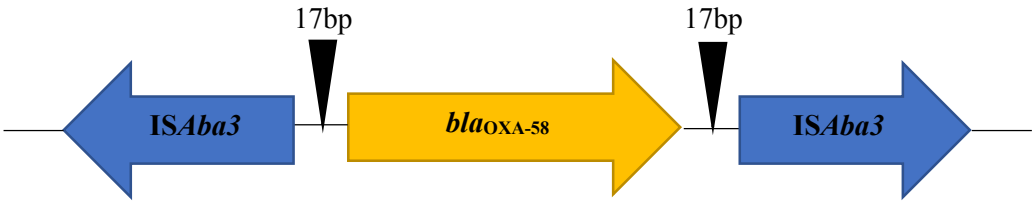
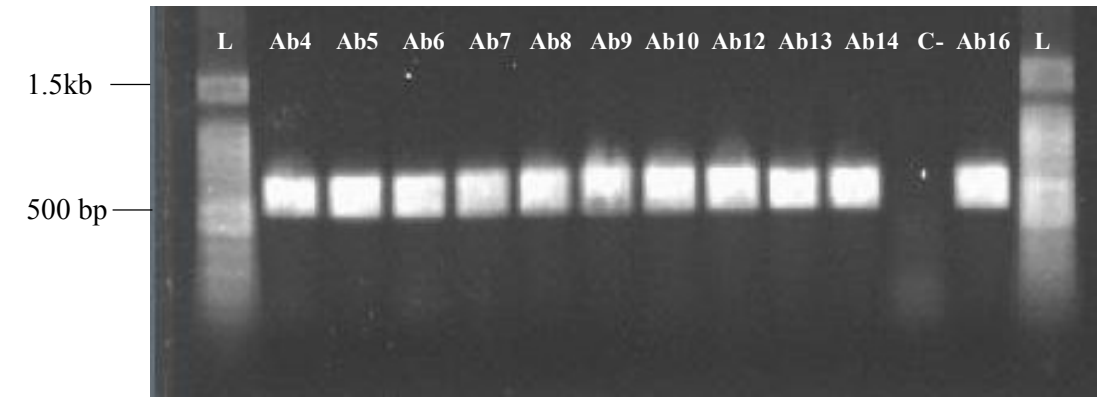


Figure 44. Genetic structure containing the *bla*$_{OXA-58}$ gene in isolates from Chile. The spacer region is indicated by the black triangles.

The presence of IS*Aba3* flanking the *bla*$_{OXA-58}$ gene was first described in 2003 in an *A. baumannii* isolate from France (Poirel *et al*., 2005b). This element is commonly associated with the *bla*$_{OXA-58}$ gene (Evans *et al*., 2013) and this association has been detected in isolates from South America, specifically, in isolates from Bolivia (Lopes *et al.*, 2013) and Argentina (Merkier *et al*., 2008). This genetic element may be playing a role in the regulation of the expression of the *bla*$_{OXA-58}$ gene, as it is capable of providing a strong promoter, which could increase the expression of the gene (Evans *et al*., 2013). In addition, this IS element may be playing an important role in the mobilisation of this carbapenemase gene, as it was found bracketing the gene, which could allow its dissemination by homologous recombination (Poirel and Nordmann, 2006).

In order to analyse the influence of the IS*Aba3* in the expression of *bla*$_{OXA-58}$, RT-PCR experiments were performed as described in Chapter 2. The expression of *bla*$_{OXA-58}$ was normalised by comparing the intensity band of the gene with the intensity band of the *16S* gene, which is a constitutive gene (Lopes *et al*., 2012b). The expression levels were compared among all the *bla*$_{OXA-58}$ positive samples as shown in the Figure 45.

A



B

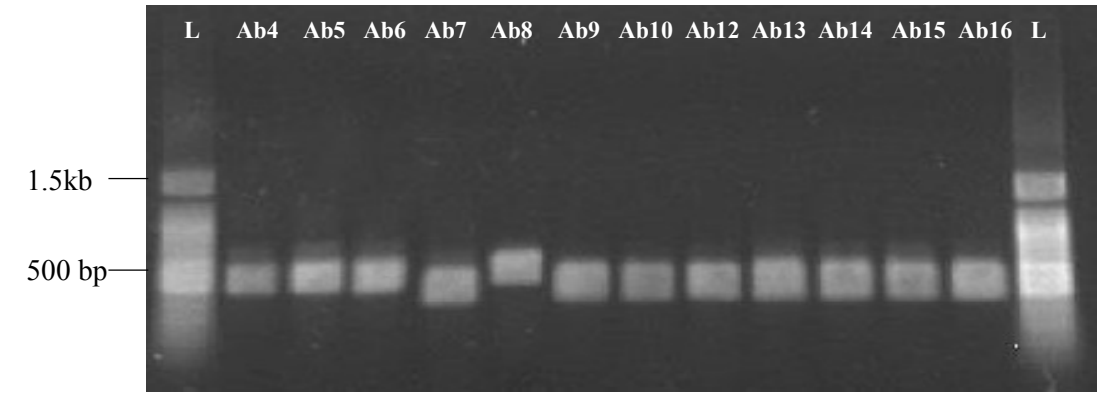


Figure 45. RT-PCR for *bla*$_{\text{OXA-58}}$ contained in the Chilean isolates. A: RT-PCR for the *bla*$_{\text{OXA-58}}$ gene. B: RT-PCR for the *16S* gene. L: 100bp DNA ladder. C-: The isolate Ab15 was used as negative control for *bla*$_{\text{OXA-58}}$.

The comparison of the intensities of the bands between samples that harboured the IS*Aba3* upstream the *bla*$_{OXA-58}$ and those where this genetic element was absent resulted in a similar expression of the carbapenemase gene (Table 9).

Table 9. Levels of gene expression in Chilean isolates of *A. baumannii*. * Isolates that harboured the IS*Aba3* upstream the *bla*$_{OXA-58}$ gene.

Strain	**Expression of *bla*$_{OXA-58}$**	**Expression of *16S***	**OXA-58/16S**
Ab4	151	106	1.42
Ab5*	160	148	1.08
Ab6*	153	112	1.36
Ab7*	148	127	1.16
Ab8*	157	113	1.39
Ab9*	159	97	1.64
Ab10	158	108	1.46
Ab12	186	101	1.84
Ab13	145	107	1.35
Ab14	159	143	1.11
Ab16*	161	119	1.35

The results presented in the Table 9 suggest that the IS*Aba3* did not produce a significant difference with the isolates that do not have this genetic element, as the levels of the expression of *bla*$_{\text{OXA-58}}$ varied between 1.08 and 1.84 (Table 9). These values could be explained if the promoter, provided by IS*Aba3,* had a similar activity in comparison with a potential promoter present in the samples that do not have this IS element. The limitation of this technique in this study is the lack of a chromosomal-positive control, which is used to normalise the intensity values. However, in the results presented in the Table 9, it is possible to presume about the role of IS*Aba3* in the expression of *bla*$_{\text{OXA-58}}$. Further analysis with extensive controls and a quantitative RT-PCR are required in order to achieve a complete understanding of IS*Aba3* in the expression of this carbapenemase gene.

It was necessary to analyse the sequence of the upstream region of the isolates that do not have the IS*Aba3* and search for the promoter regions, as probably there are other genetic arrangements that are regulating the expression of this gene. In this sense, there have been different genetic arrangements involving the presence of IS*Aba3*, such as the insertion of IS*Aba2* into the IS*Aba3* element in the same orientation of the *bla*$_{\text{OXA-58}}$ gene, producing an hybrid promoter where the -35 region belongs to the IS*Aba2* element and the -10 region to IS*Aba3* (Poirel and Nordmann, 2006). Furthermore, the IS*18* has been detected inserted into the IS*Aba3* element, forming a chimeric promoter that controls the expression of *bla*$_{\text{OXA-58}}$ (Poirel and Nordmann, 2006). Another re-arrangement has been represented by the insertion of the IS*Aba825* into the IS*Aba3*, which results in enhanced levels of carbapenem resistance (Ravasi *et al*., 2011). The insertion of IS*1008* or IS*Aba9* into IS*Aba3* also could produce hybrid promoters that result in the increased expression of the *bla*$_{\text{OXA-58}}$ gene (Chen *et al*., 2008; Figueiredo *et al*., 2009b).

In addition to controlling the expression of carbapenemase genes, the IS elements may have an important role in the duplication of these genes. According to a study published in 2007, three clonal isolates with MICs of imipenem of 16, 32 and 128mg/l, harboured the *bla*$_{\text{OXA-58}}$. Interestingly, the samples with a MIC of 16mg/l had one copy of *bla*$_{\text{OXA-58}}$, while the one that had a MIC of 32mg/l had two copies and the one with a MIC of 128mg/l contained three copies of the carbapenemase gene (Bertini *et al*., 2007).

By analysing the genetic context of the *bla*$_{\text{OXA-58}}$ gene, the IS*aba2*/IS*Aba3*-*bla*$_{\text{OXA-58}}$-IS*Aba3* arrangement was flanked by copies of the IS*26* element, which could be involved in production of multiple copies of *bla*$_{\text{OXA-58}}$. This effect of IS elements in achieving the duplication of carbapenemases genes is important to investigate as most of the Chilean samples harboured more than one copy of *bla*$_{\text{OXA-58}}$, which could be produced by a similar mechanism to that proposed by Bertini *et al* (2007).

This is the first report of the IS*Aba3* associated with *bla*$_{\text{OXA-58}}$ in isolates from Chile, though IS*Aba3* has been found associated with *bla*$_{\text{OXA-58}}$ elsewhere (reviewed by Evans *et al*., 2013). The lack of national data on the mechanisms of carbapenem resistance in *A. baumannii* in Chile makes it imperative to work on the characterisation of the IS elements that govern the mobilisation and expression of the carbapenemases genes.

IS*Aba3* may be playing a role in the mobilisation of the *bla*$_{\text{OXA-58}}$ gene in the isolates that contained this arrangement (Figure 44), as the carbapenemase gene is contained in a transposon-like structure. The presence of the IS*Aba3* element, associated with the *bla*$_{\text{OXA-58}}$ carbapenemase gene, has been detected in isolates from South America, specifically in isolates from Bolivia, where it increased the expression of the *bla*$_{\text{OXA-58}}$ carbapenemase gene

(Lopes *et al*., 2013). In turn, this genetic arrangement was found in a plasmid of ca. 40kb, which provides the possibility to spread this carbapenemase gene among different pathogens.

Interestingly, this genetic arrangement was also identified in a isolate of *A. pittii* (formerly *Acinetobacter* genomic species 3) with reduced susceptibility to carbapenems, where IS*Aba3* was present downstream the $bla_{\text{OXA-58}}$ gene and a truncated copy, which was interrupted by IS*Aba125*, was located upstream the carbapenemase gene (Evans *et al*., 2010). This structure may account for the potential dissemination of the $bla_{\text{OXA-58}}$ gene between different species.

3.5. To characterise the mechanism of ceftazidime-resistance in *A. baumannii* isolates collected in 2008 from Al Ain, UAE.

As described earlier in this thesis, the two isolates (NM55 and NM128) obtained from the UAE corresponded to isogenic strains as they exhibited a similarity higher than 90% by PFGE (Figure 35) but they showed a marked difference in their MICs values of ceftazidime. Furthermore, as described above, the MICs of rifampicin were 2.0mg/l for isolate NM128 and 32mg/l for isolate NM55. These two MDR-isolates were collected in May (NM55) and August (NM128) 2008 from tracheal specimens of a 6-years-old patient in Tawam Hospital, Al Ain, UAE. As stated above, the MICs values for ceftazidime were 64mg/l for isolate NM55 and 0.2mg/l for the isolate NM128.

In order to characterise the mechanism of ceftazidime-resistance in these samples, a set of PCRs were performed as described in the Chapter 2. As the main mechanism of ceftazidime-resistance in *A. baumannii* is usually represented by the increased expression of the intrinsic

bla$_{ADC}$ genes, this mechanism was investigated first. The increase of the expression of *bla*$_{ADC}$ depends of the presence of genetic elements, such as IS elements, present upstream these genes (Ruiz *et al.*, 2007). The sequencing of the *bla*$_{ADC}$ genes revealed that both isolates harboured the ADC-26 variant.

The primers utilised in the characterisation of the upstream region of the *bla*$_{ADC}$ genes aligned in a conserved gene present further upstream, the GTP cyclohydrolase I gene, thus allowing the detection any IS present immediately upstream. If there was no IS present upstream of the β-lactamase gene a PCR product of 360bp was obtained. As this was the case, as shown in Figure 46, there were no IS elements present upstream the *bla*$_{ADC}$ genes.

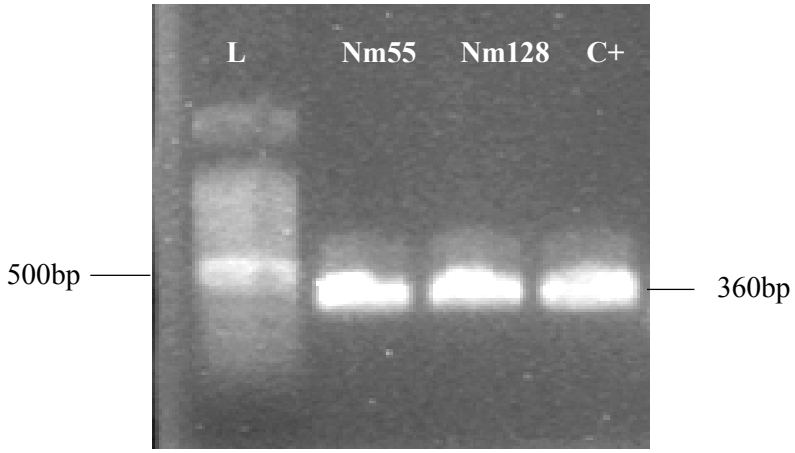


Figure 46. Detection of ISs upstream the *bla*$_{ADC}$ genes. C+: DNA extracted from *A. baumannii* ATCC19606 used as positive control. L: 100bp DNA ladder.

After the characterisation of the upstream region of the *bla*$_{ADC}$, where both isolates presented an identical conformation, the presence of ESBLs was performed by PCR, in order to explain the difference of the susceptibility patterns to ceftazidime. As presented in Figure 47, the isolate NM55 was positive for ESBLs of the PER family.

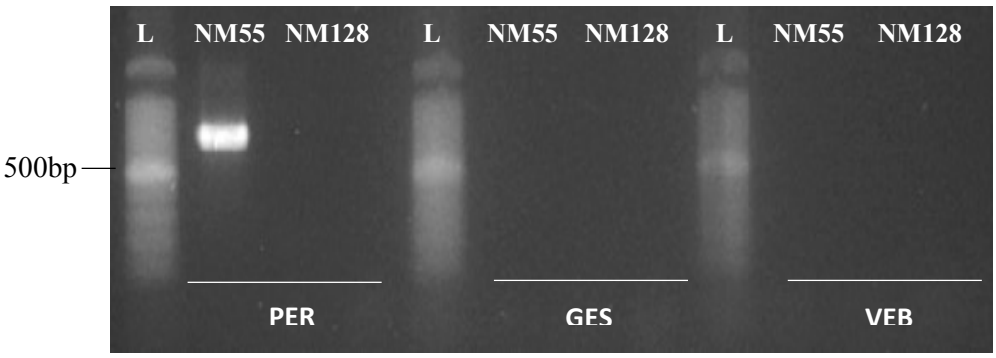


Figure 47. PCR-screening of PER, GES and VEB ESBLs in the isolates from the UAE. L: 100bp DNA ladder.

Through sequencing of the PCR products obtained, the *bla*$_{\text{PER-like}}$ gene was identified as *bla*$_{\text{PER-7}}$ (Figure 48).

Download GenPept Graphics

blaPER-7 [Acinetobacter baumannii]

Sequence ID: gb|AEI54993.1| Length: 308 Number of Matches: 1

See 1 more title(s)

Range 1: 1 to 308 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
629 bits(1623)	0.0	Compositional matrix adjust.	308/308(100%)	308/308(100%)	0/308(0%)

```
Query  1    MNVIIKAVVTASTLLMVSFSSFETSAQSPLLKEQIESIVIGKKATVGVAVWGPDDLEPLL  60
            MNVIIKAVVTASTLLMVSFSSFETSAQSPLLKEQIESIVIGKKATVGVAVWGPDDLEPLL
Sbjct  1    MNVIIKAVVTASTLLMVSFSSFETSAQSPLLKEQIESIVIGKKATVGVAVWGPDDLEPLL  60

Query  61   INPFEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQTVIVNRAKVLQNTWAPIMKAYQGDQF  120
            INPFEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQTVIVNRAKVLQNTWAPIMKAYQGDQF
Sbjct  61   INPFEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQTVIVNRAKVLQNTWAPIMKAYQGDQF  120

Query  121  SVPVQQLLQYSVSHSDNVACDLLFELVGGPAALHDYIQSMGIKETAVVANEAQMHADDQV  180
            SVPVQQLLQYSVSHSDNVACDLLFELVGGPAALHDYIQSMGIKETAVVANEAQMHADDQV
Sbjct  121  SVPVQQLLQYSVSHSDNVACDLLFELVGGPAALHDYIQSMGIKETAVVANEAQMHADDQV  180

Query  181  QYQNWTSMKGAAEILKKFEQKTQLSETSQALLWKWMVETTTGPERLKGLLPAGTVVAHKT  240
            QYQNWTSMKGAAEILKKFEQKTQLSETSQALLWKWMVETTTGPERLKGLLPAGTVVAHKT
Sbjct  181  QYQNWTSMKGAAEILKKFEQKTQLSETSQALLWKWMVETTTGPERLKGLLPAGTVVAHKT  240

Query  241  GTSGVRAGKTAATNDLGIILLPDGRPLLVAVFVKDSAESSRTNEAIIAQVAQAAYQFELK  300
            GTSGVRAGKTAATNDLGIILLPDGRPLLVAVFVKDSAESSRTNEAIIAQVAQAAYQFELK
Sbjct  241  GTSGVRAGKTAATNDLGIILLPDGRPLLVAVFVKDSAESSRTNEAIIAQVAQAAYQFELK  300

Query  301  KLSALSPN  308
            KLSALSPN
Sbjct  301  KLSALSPN  308
```

Figure 48. Comparison of the amino acid sequence of the *bla*$_{\text{PER-like}}$ present in the isolate NM55 with the sequence available in the NCBI database (http://www.ncbi.nlm.nih.gov/).

The PER-like enzymes previously described in *A. baumannii* were predominantly PER-1 and PER-2, which have been identified in isolates from different continents (reviewed by Peleg *et al*., 2008). An isolate collected in 2013 from Kuwait from a 75-years old patient, contained the PER-1 enzyme, reflecting the presence of this enzyme group in the Middle East. Interestingly, the genetic environment of the gene comprised two copies of the IS*Pa12* element, which were flanking the $bla_{\text{PER-1}}$ gene and this genetic structure was also plasmid located (Opazo *et al*., 2014). The activity of the copies of the IS*Pa12*, either side of the $bla_{\text{PER-1}}$ gene, could facilitate the spread of this gene. Additionally in this isolate, there was an extra mechanism of ceftazidime-resistance, represented by the presence of an IS*Aba1* element upstream the bla_{ADC} gene, which may account, together with PER-1, for the high MIC value of ceftazidime, which was >256mg/l (Opazo *et al*., 2014).

This work reconfirmed the remarkable capacity of *A. baumannii* to capture and express genes of antibiotic resistance, representing one of the most important factors transforming this microbe into a persistent and dangerous nosocomial pathogen.

PER-7 enzyme was first characterised in 2011. This enzyme was present in an isolate collected from bronchial aspirate in 2010 from a 30-years old patient hospitalised in Paris, France. Compared with PER-1, PER-7 had four aminoacid substitutions, Q119E, V245I, R246K and A294T, being more similar to PER-6, which had just one different amino acid (Bonnin *et al*., 2011c). PER-7 possess a higher activity against ceftazidime, compared with PER-6, whereas, its activity against carbapenems is lower when compared with PER-6 (Bonnin *et al*., 2011c).

Interestingly, both samples, NM55 and NM128, harboured a large plasmid (Figure 49). The plasmid extraction and hybridization experiments were performed in the laboratory of Professor Tibor Pál, in the UAE. The plasmids sizes were determined by S1 nuclease digestion, which showed that the NM55 contained a plasmid of ca. 200kb whereas the NM128 harboured a plasmid of ca. 180kb. According to the Southern blotting experiment (Figure 49), it was determined that the *bla*$_{\text{PER-7}}$ gene was located on the large plasmid present in the NM55 strain, but not in the plasmid detected in the NM128. Additionally, the plasmid from NM55 was not conjugative, irrespective of the recipient utilised. Unlike the previous detection of this gene in the French isolate, where it was located in the chromosome (Bonnin *et al*., 2011c), the *bla*$_{\text{PER-7}}$ from NM55 was located on a plasmid, although without an IS element upstream to provide a surrogate promoter which could control its expression.

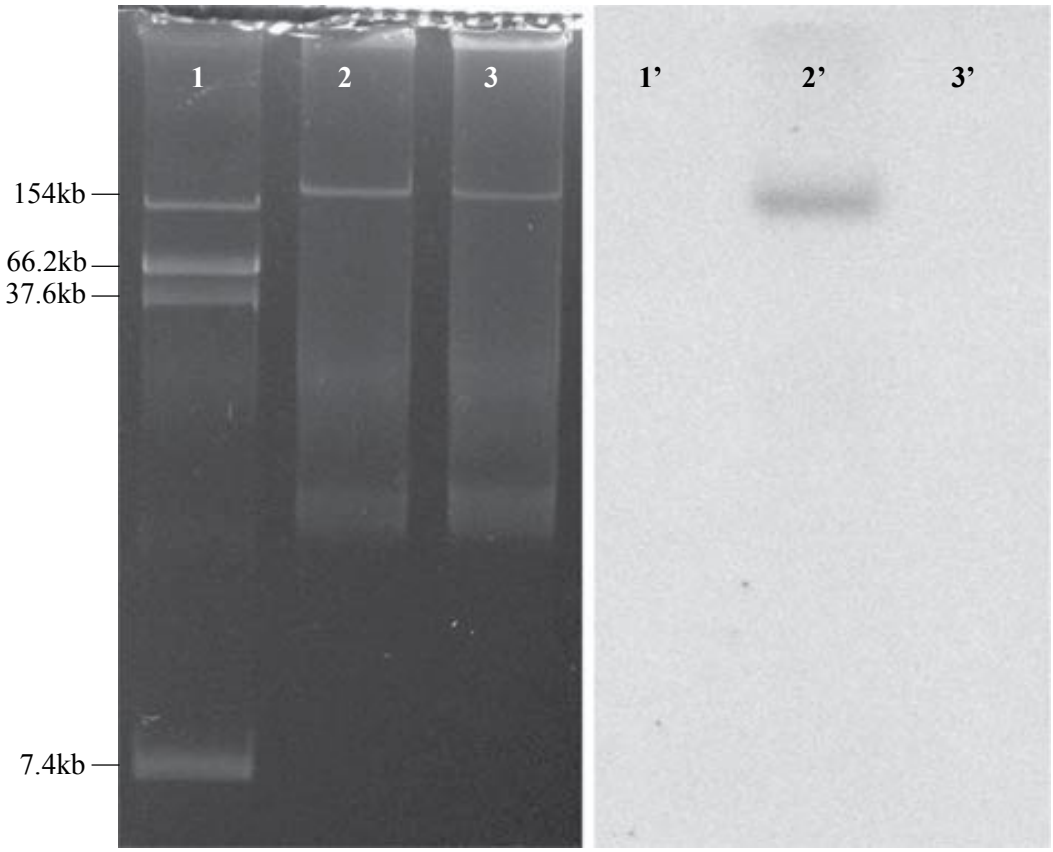


Figure 49. Southern blot of the plasmids extracted from *A. baumannii* strains NM55 and NM128 hybridised with a bla_{PER-7} probe. The gel on the left shows the plasmid profiles obtained by the S1 digestion while the gel on the right shows the hybridisation with the specific probe. 1: Ladder 39R861MW; 2: NM55; 3: NM128.

The first report of PER-7 identified the gene was located on the chromosome and associated it with a mosaic class 1 integron structure, which belongs to the IS*CR1*-like family (Bonnin *et al*., 2011c). In order to characterise the genetic platform that contains the PER-7 in the NM55, a series of PCRs with different primers were performed as described in the Chapter 2.

The amplification and sequencing showed that, as in the case of the French isolate, the *bla*$_{PER-7}$ was contained in a class 1 integron structure, which also contained the *arr-2* and *cmlA7* genes, conferring resistance to rifampicin and chloramphenicol, respectively (Figure 50).

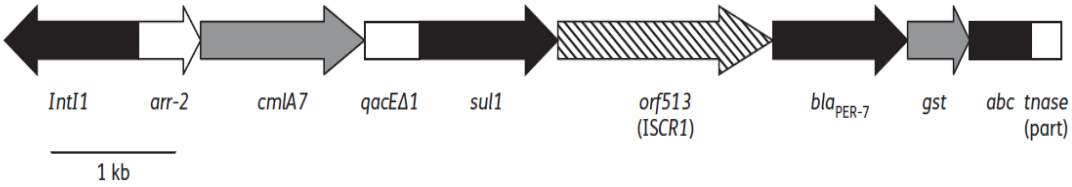


Figure 50. Schematic representation of the genetic environment of the *bla*$_{PER-7}$ gene in strain NM55. Horizontal arrows represent the genetic orientations. Partial genes are represented by squares. *tnase*, transposase. The *orf513* gene, which determines the IS*CR1*-element, is highlighted by diagonal lines.

Upstream the *bla*$_{PER-7}$ was the 3'-CS region of the class 1 integron that contained the *qacEΔ1* and *sul1* genes, but the *orf5*, which is part of the classic structure of these genetic elements, was absent (Figure 50). Immediately downstream of the *bla*$_{PER-7}$ gene was a *gst* gene. Interestingly, further downstream there was a portion of an *abc* transporter and then a transposase gene.

The genetic context in which the *bla*$_{PER-7}$ from NM55 differed from the chromosomally-located allele in the French isolate specifically in the downstream region (Figure 51). In the case of the French isolate, the *ABAYE3396* gene was located downstream, while in the case of the NM55 strain, the *gst* gene and a part of an *abc* transporter were located in this region.

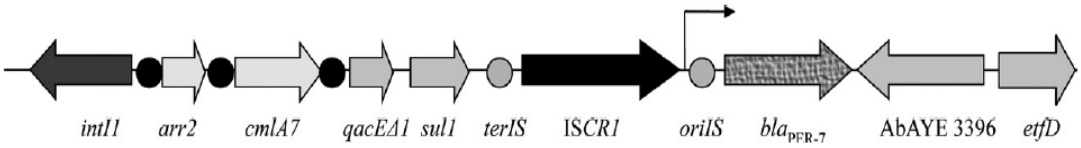


Figure 51. Schematic map representing the genetic environment surrounding the *bla*$_{PER-7}$ in the AP2 strain from France (Bonnin *et al*., 2011c).

This IS*CR1* structure has been identified in different species. Specifically, it was described in *Aeromonas punctata* isolated in China (Xia *et al*., 2010). In this case, the *bla*$_{PER-1}$ was located in this genetic platform. This could indicate that probably this genetic structure originated in a different bacterial species.

As described above, the downstream region in the NM55 strain was flanked by an *abc* transporter gene, which was interrupted by a transposase gene. This indicates a probable insertion of the IS*CR1* element in this specific locus, which may suggest a hot spot for the integration of mobile genetic elements. In addition, the variable zone of the mosaic class 1 integron in the UAE strain NM55 contained the *arr-2* gene, which explains the reduced susceptibility to rifampicin, in comparison with the NM128 strain (Figure 50). Moreover, the NM55 strain contained the *cmlA7* gene, which confers resistance to chloramphenicol; however, both strains were resistant to this antibiotic, suggesting that the mechanism of resistance was not due only by the presence of this gene.

Despite the previously described differences, there are some similarities between the French strain AP2 and NM55. Both samples harboured the same *bla*$_{OXA-51-like}$ gene, namely *bla*$_{OXA-64}$,

which is one of the most common genes of this family present in the Middle East (Opazo *et al*., 2012a). It is notable that the French isolate was collected in 2010 while the NM55 strain was isolated about two years earlier. This chronology may suggest that the *bla*$_{PER-7}$ was originally carried into strains of *A. baumannii* on plasmids, and probably some of the integrons were mobilised to the chromosome. In the results presented in this thesis, the plasmid was not conjugative, thus the mechanism of its uptake is still to be analysed.

These results showed once more the remarkably ability of *A. baumannii* to capture and/or lose genes, during which process elements, such as IS*CR1,* could be involved. Interestingly, in the case of the strains from the UAE, the *bla*$_{PER-7}$ was lost after four months, leaving the resulting strain, NM128, susceptible to ceftazidime. The reasons of this loss are not easy to explain in an environment where cephalosporins are being used frequently.

Finally, the NM55 and NM128 strains derived from the same patient, showing PFGE patterns of more than 90% similarity, which suggests that the smaller plasmid (NM128) derived from the plasmid contained in the NM55 strain, which had a suffered a deletion, including the *bla*$_{PER-7}$ gene.

Chapter 4 Conclusions

The results obtained in this thesis showed that the Mobilome is playing an important role in the ceftazidime- and carbapenem-resistance in MDR-*A. baumannii* from Chile and the UAE.

In the case of the Chilean isolates, the Mobilome was composed by plasmids harbouring the *bla*$_{\text{OXA-58}}$ enzyme in 12 of the 15 samples analysed. In turn, another member of the Mobilome, the IS*Aba3*-element, was associated with *bla*$_{\text{OXA-58}}$, forming a transposon-like structure. Even though the results need to be validated, they show that IS*Aba3* would not be involved in the expression of *bla*$_{\text{OXA-58}}$. In addition, another IS element, IS*Aba1* was present in the Chilean carbapenem-resistant strains that do not harbour the acquired *bla*$_{\text{OXA-58}}$. This element was located upstream the *bla*$_{\text{OXA-51-like}}$ genes, which may be causing the over-expression of these carbapenemases genes. On the other hand, it is necessary to characterised the isolates by MLST in order to determine whether they are part of the 3 widely spread ICs or whether they belong to different local lineages.

In the case of the Emirati isolates, the resistance to ceftazidime was mediating by the Mobilome, which consisted of plasmids harbouring the IS*CR1*-element, which, in turn, contained the uncommon *bla*$_{\text{PER-7}}$ ESBLs gene. The structure of this genetic platform represents a new genetic context harbouring this gene, which reflects the remarkable ability of *A. baumannii* to capture and express genes making it a pathogen with a high ability to survive under adverse conditions. Additionally, the IS*CR1*-element harboured two extra resistance genes, such as *arr-2* and *clmA7*, conferring resistance to more than a specific group of antibiotics.

To conclude, the role of the Mobilome in the strains analysed is to spread and to regulate the expression of resistance genes in *A. baumannii* by the presence of several members of this group, such as ISs, transposons-like structures, plasmids and the IS*CR1*-elements. The results of this thesis suggest that MDR-*A. baumannii* isolates can act as reservoir of resistance genes, where elements belonging to the Mobilome could mediate the mobilisation from resistance to susceptible cells, which might produce a widely appearance of MDR isolates, provoking a serious public health problem.

References

Acosta MB, Ferreira RC, Padilla G, *et al*. (2000) Altered expression of oligopeptide-binding protein (OppA) and aminoglycoside resistance in laboratory and clinical *Escherichia coli* strains. *Journal of medical microbiology*, 49, 409–13.

Adams MD, Goglin K, Molyneaux N, *et al*. (2008) Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *Journal of bacteriology*, 190, 8053–64.

Adams MD, Nickel GC, Bajaksouzian S, *et al*. (2009) Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrobial agents and chemotherapy*, 53, 3628–34.

Al-Kaabi MR, Tariq WU-Z and Hassanein AA (2011) Rising bacterial resistance to common antibiotics in Al Ain, United Arab Emirates. *Eastern Mediterranean health journal,* 17, 479–84.

Alós J-I (2009) [Quinolones]. *Enfermedades infecciosas y microbiología clínica*, 27, 290–7,

Aminov RI (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology*, 1, 134.

Amyes SG and Smith JT (1974) R-factor trimethoprim resistance mechanism: an insusceptible target site. *Biochemical and biophysical research communications*, 58, 412–8.

Andersson DI and Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews. Microbiology*, Nature Publishing Group, 8, 260–71.

Andrews J (2010) BSAC Methods for Antimicrobial Susceptibility Testing.

Andriole VT (2005) The quinolones: past, present, and future. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 41 Suppl 2, S113–9.

Anstey NM, Currie BJ, Hassell M, *et al*. (2002) Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with carriage in the throat in at-risk groups. *Journal of clinical microbiology*, 40, 685–6.

Antunes P, Machado J, Sousa JC, *et al*. (2005) Dissemination of sulfonamide resistance genes (*sul1, sul2*, and *sul3*) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrobial agents and chemotherapy*, 49, 836–9.

Avery OT, Macleod CM and McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from *Pneumococcus* type III. *The Journal of experimental medicine*, 79, 137–58.

Bae IK, Lee Y-N, Lee WG, *et al*. (2007) Novel complex class 1 integron bearing an IS*CR1* element in an *Escherichia coli* isolate carrying the *bla*$_{\text{CTX-M-14}}$ gene. *Antimicrobial agents and chemotherapy*, 51, 3017–9.

Bartual SG, Seifert H, Hippler C, *et al*. (2005) Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *Journal of clinical microbiology*, 43, 4382–90.

Bassetti M, Repetto E, Righi E, *et al*. (2008) Colistin and rifampicin in the treatment of multidrug-resistant *Acinetobacter baumannii* infections. *The Journal of antimicrobial chemotherapy*, 61, 417–20.

Baumann P, Doudoroff M and Stanier RY (1968) A study of the *Moraxella* group. II. Oxidative-negative species (genus *Acinetobacter*). *Journal of bacteriology*, 95, 1520–41.

Begg EJ and Barclay ML (1995) Aminoglycosides--50 years on. *British journal of clinical pharmacology*, 39, 597–603.

Bergogne-Bérézin E and Towner KJ (1996) *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clinical microbiology reviews*, 9, 148–65.

Bertini A, Poirel L, Bernabeu S, *et al*. (2007) Multicopy *bla*$_{\text{OXA-58}}$ gene as a source of high-level resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 51, 2324–8.

Bertini A, Poirel L, Mugnier PD, *et al*. (2010) Characterization and PCR-based replicon typing of resistance plasmids in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 54, 4168–77.

Bhagan-Bruno S, Lather N and Fergus I V (2010) *Acinetobacter* endocarditis presenting as a large right atrial mass: an atypical presentation. *Echocardiography*, 27, E39–42.

Bijie H, Kulpradist S, Manalaysay M, *et al*. (2005) In vitro activity, pharmacokinetics, clinical efficacy, safety and pharmacoeconomics of ceftriaxone compared with third and fourth generation cephalosporins: review. *Journal of chemotherapy*, 17, 3–24.

Blondeau JM (2004) Fluoroquinolones: mechanism of action, classification, and development of resistance. *Survey of ophthalmology*, 49 Suppl 2, S73–8.

Bo G (2000) Giuseppe Brotzu and the discovery of cephalosporins. *Clinical microbiology and infection*, 6 Suppl 3, 6–8.

Bogaerts P, Naas T, Wybo I, *et al*. (2006) Outbreak of infection by carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-58 in Belgium. *Journal of clinical microbiology*, 44, 4189–92.

Bonnet R, Marchandin H, Chanal C, *et al*. (2002) Chromosome-encoded class D beta-lactamase OXA-23 in *Proteus mirabilis*. *Antimicrobial agents and chemotherapy*, 46, 2004–6.

Bonnin R A, Poirel L, Licker M, *et al*. (2011a) Genetic diversity of carbapenem-hydrolysing β-lactamases in *Acinetobacter baumannii* from Romanian hospitals. *Clinical microbiology and infection*, 17, 1524–8.

Bonnin Rémy A, Nordmann P, Potron A, *et al*. (2011b) Carbapenem-hydrolyzing GES-type extended-spectrum beta-lactamase in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 55, 349–54.

Bonnin Rémy A, Potron A, Poirel L, *et al*. (2011c) PER-7, an extended-spectrum beta-lactamase with increased activity toward broad-spectrum cephalosporins in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 55, 2424–7

Bonnin RA, Rotimi VO, Al Hubail M, *et al*. (2013) Wide dissemination of GES-type carbapenemases in *Acinetobacter baumannii* isolates in Kuwait. *Antimicrobial agents and chemotherapy*, 57, 183–8.

Bosch F and Rosich L (2008) The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. *Pharmacology*, 82, 171–9.

Bou G, Oliver A and Martínez-Beltrán J (2000) OXA-24, a novel class D beta-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrobial agents and chemotherapy*, 44, 1556–61.

Brisou J and Prevot AR (1954) [Studies on bacterial taxonomy. X. The revision of species under *Acromobacter* group]. *Annales de l'Institut Pasteur*, 86, 722–8.

Brown S and Amyes SGB (2005) The sequences of seven class D beta-lactamases isolated from carbapenem-resistant *Acinetobacter baumannii* from four continents. *Clinical microbiology and infection*, 11, 326–9.

Brown S, Young HK and Amyes SGB (2005) Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. *Clinical microbiology and infection*, 11, 15–23.

Bryskier A (1998) Novelties in the field of anti-infectives in 1997. *Clinical infectious diseases,* 27, 865–83

Bush K (1989) Characterization of beta-lactamases. *Antimicrobial agents and chemotherapy*, 33, 259–63

Bush K (2010) Bench-to-bedside review: The role of beta-lactamases in antibiotic-resistant Gram-negative infections. *Critical care*, 14, 224.

Bush K and Jacoby GA (2010) Updated functional classification of beta-lactamases. *Antimicrobial agents and chemotherapy*, 54, 969–76.

Bush K and Mobashery S (1998) How beta-lactamases have driven pharmaceutical drug discovery. From mechanistic knowledge to clinical circumvention. *Advances in experimental medicine and biology*, 456, 71–98.

Bush K, Jacoby GA and Medeiros AA (1995) A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial agents and chemotherapy*, 39, 1211–33.

Cambray G, Guerout A-M and Mazel D (2010) Integrons. *Annual review of genetics*, 44, 141–66.

Campbell EA, Korzheva N, Mustaev A, *et al*. (2001) Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell*, 104, 901–12.

Carbon C (1995) Clinical relevance of intracellular and extracellular concentrations of macrolides. *Infection*, 23 Suppl 1, S10–4.

Caroff N, Espaze E, Gautreau D, *et al*. (2000) Analysis of the effects of -42 and -32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing ampC. *The Journal of antimicrobial chemotherapy*, 45, 783–8.

Chen T-L, Wu RC-C, Shaio M-F, *et al*. (2008) Acquisition of a plasmid-borne $bla_{\text{OXA-58}}$ gene with an upstream IS*1008* insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 52, 2573–80.

Chen T-L, Lee Y-T, Kuo S-C, *et al*. (2010) Emergence and Distribution of Plasmids Bearing the $bla_{\text{OXA-51-like}}$ gene with an upstream IS*Aba1* in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. *Antimicrobial agents and chemotherapy*, 54, 4575–81.

Choi CH, Hyun SH, Lee JY, *et al*. (2008) *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cellular microbiology*, 10, 309–19.

Choi S-H, Choo EJ, Kwak YG, *et al*. (2006) Clinical characteristics and outcomes of bacteremia caused by *Acinetobacter* species other than *A. baumannii*: comparison with A. *baumannii* bacteremia. *Journal of infection and chemotherapy*, 12, 380–6.

Clatworthy AE, Pierson E and Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nature chemical biology*, 3, 541–8.

Colabella J and Chagan L (2008) Dalbavancin (zeven), a novel glycopeptide for resistant gram-positive organisms. *Pharmacy & Therapeutics*, 33, 42–57.

Conway SP, Pond MN, Watson A, *et al*. (1997) Intravenous colistin sulphomethate in acute respiratory exacerbations in adult patients with cystic fibrosis. *Thorax*, 52, 987–93

Corvec S, Caroff N, Espaze E, *et al*. (2003) AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *The Journal of antimicrobial chemotherapy*, 52, 629–35.

Coyne S, Rosenfeld N, Lambert T, *et al*. (2010) Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 54, 4389–93.

Del Mar Tomás M, Beceiro A, Pérez A, *et al*. (2005) Cloning and functional analysis of the gene encoding the 33- to 36-kilodalton outer membrane protein associated with

carbapenem resistance in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 49, 5172–5.

Delbrück H, Bogaerts P, Kupper MB, *et al*. (2012) Kinetic and crystallographic studies of extended-spectrum GES-11, GES-12, and GES-14 β-lactamases. *Antimicrobial agents and chemotherapy*, 56, 5618–25.

Derbyshire KM, Hwang L and Grindley ND (1987) Genetic analysis of the interaction of the insertion sequence IS*903* transposase with its terminal inverted repeats. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 8049–53.

Diancourt L, Passet V, Nemec A, *et al*. (2010) The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. *PloS one*, 5, e10034.

Dijkshoorn L, Nemec A and Seifert H (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature reviews. Microbiology*, 5, 939–51.

Diomedi P A (2005) Infecciones por *Acinetobacter baumannii* pan-resistente: Consideraciones epidemiológicas y de manejo antimicrobiano actualizado. *Revista chilena de infectología*, Sociedad Chilena de Infectología, 22, 298–320.

Dobrindt U, Hochhut B, Hentschel U, *et al*. (2004) Genomic islands in pathogenic and environmental microorganisms. *Nature reviews. Microbiology*, 2, 414–24.

Doi Y, Wachino J-I, Yamane K, *et al*. (2004) Spread of novel aminoglycoside resistance gene aac(6')-Iad among *Acinetobacter* clinical isolates in Japan. *Antimicrobial agents and chemotherapy*, 48, 2075–80.

Dolzani L, Tonin E, Lagatolla C, *et al*. (1995) Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences. *Journal of clinical microbiology*, 33, 1108–13

Domagk G (1957) Twenty-five years of sulfonamide therapy. *Annals of the New York Academy of Sciences*, 69, 380–4.

Donald HM, Scaife W, Amyes SG, *et al*. (2000) Sequence analysis of ARI-1, a novel OXA beta-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrobial agents and chemotherapy*, 44, 196–9.

Dorsey CW, Beglin MS and Actis LA (2003) Detection and analysis of iron uptake components expressed by *Acinetobacter baumannii* clinical isolates. *Journal of clinical microbiology*, 41, 4188–93.

Douthwaite S and Champney WS (2001) Structures of ketolides and macrolides determine their mode of interaction with the ribosomal target site. *Journal of Antimicrobial Chemotherapy*, 48 Suppl 2, 1–8.

Duggar BM (1948) Aureomycin: A product of the continuing search for new antibiotics. *Annals of the New York Academy of Sciences*, 51, 177–181

Duma RJ (1988) Microbiology and pharmacology of aztreonam. *Urology*, 31 Suppl 6, 9–13.

Duplessis C and Crum-Cianflone NF (2011) Ceftaroline: A New Cephalosporin with Activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Clinical medicine reviews in therapeutics*, 10, 3.

Durante-Mangoni E and Zarrilli R (2011) Global spread of drug-resistant *Acinetobacter baumannii*: molecular epidemiology and management of antimicrobial resistance. *Future microbiology*, 6, 407–22.

Ecker JA, Massire C, Hall TA, *et al*. (2006) Identification of *Acinetobacter* species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. *Journal of clinical microbiology*, 44, 2921–32.

Ehrenstein B, Bernards AT, Dijkshoorn L, *et al*. (1996) *Acinetobacter* species identification by using tRNA spacer fingerprinting. *Journal of clinical microbiology*, 34, 2414–20.

Enright MC and Spratt BG (1999) Multilocus sequence typing. *Trends in microbiology*, 7, 482–7.

Erridge C, Moncayo-Nieto OL, Morgan R, *et al*. (2007) *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling. *Journal of medical microbiology*, 56, 165–71.

Eshraghi SS, Heidarzadeh S, Soodbakhsh A, *et al*. (2014) Pulmonary nocardiosis associated with cerebral abscess successfully treated by co-trimoxazole: a case report. *Folia microbiologica*, 59, 277-81.

Espinal P, Poirel L, Carmeli Y, *et al*. (2013) Spread of NDM-2-producing *Acinetobacter baumannii* in the Middle East. *The Journal of antimicrobial chemotherapy*, 68, 1928–30.

Evans BA, Hamouda A, Towner KJ, *et al*. (2008) OXA-51-like beta-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*. *Clinical microbiology and infection*, 14, 268–75.

Evans BA, Hamouda A, Towner KJ, *et al*. (2010) Novel genetic context of multiple $bla_{\text{OXA-58}}$ genes in *Acinetobacter* genospecies 3. *The Journal of antimicrobial chemotherapy*, 65, 1586–8.

Evans BA, Hamouda A and Amyes SGB (2013) The rise of carbapenem-resistant *Acinetobacter baumannii*. *Current pharmaceutical design*, 19, 223–38.

Falagas ME and Kasiakou SK (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clinical infectious diseases*, 40, 1333–41.

Falagas ME, Bliziotis IA and Siempos II (2006) Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Critical care*, 10, R48.

Figueiredo S, Poirel L, Croize J, *et al*. (2009a) In vivo selection of reduced susceptibility to carbapenems in *Acinetobacter baumannii* related to IS*Aba1*-mediated overexpression of the natural bla(OXA-66) oxacillinase gene. *Antimicrobial agents and chemotherapy*, 53, 2657–9.

Figueiredo S, Poirel L, Papa A, *et al*. (2009b) Overexpression of the naturally occurring $bla_{\text{OXA-51}}$ gene in *Acinetobacter baumannii* mediated by novel insertion sequence IS*Aba9*. *Antimicrobial agents and chemotherapy*, 53, 4045–7.

Fleming A (1929) On the Antibacterial Action of Cultures of a *Penicillium*, with Special Reference to their Use in the Isolation of *B. influenzæ*. *British journal of experimental pathology*, p. 226-7.

Fluit AC, Florijn A, Verhoef J, *et al*. (2005) Presence of tetracycline resistance determinants and susceptibility to tigecycline and minocycline. *Antimicrobial agents and chemotherapy*, 49, 1636–8.

Ford PJ and Avison MB (2004) Evolutionary mapping of the SHV beta-lactamase and evidence for two separate IS*26*-dependent bla_{SHV} mobilization events from the *Klebsiella pneumoniae* chromosome. *The Journal of antimicrobial chemotherapy*, 54, 69–75.

Forrest GN and Tamura K (2010) Rifampin combination therapy for nonmycobacterial infections. *Clinical microbiology reviews*, 23, 14–34.

Fournier P-E, Vallenet D, Barbe V, *et al*. (2006) Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS genetics*, 2, e7.

Furesz S (1970) Chemical and biological properties of rifampicin. *Antibiotica et chemotherapia. Fortschritte. Advances. Progrès*, 16, 316–51.

Gales AC, Jones RN and Sader HS (2006) Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of Gram-negative bacilli: report from the SENTRY antimicrobial surveillance programme (2001-2004). *Clinical microbiology and infection,* 12, 315–21.

Gales AC, Castanheira M, Jones RN, *et al*. (2012) Antimicrobial resistance among Gram-negative bacilli isolated from Latin America: results from SENTRY Antimicrobial Surveillance Program (Latin America, 2008-2010). *Diagnostic microbiology and infectious disease*, 73, 354–60.

Garnacho J, Sole-Violan J, Sa-Borges M, *et al*. (2003) Clinical impact of pneumonia caused by *Acinetobacter baumannii* in intubated patients: a matched cohort study. *Critical care medicine*, 31, 2478–82.

Garnacho-Montero J and Amaya-Villar R (2010) Multiresistant *Acinetobacter baumannii* infections: epidemiology and management. *Current opinion in infectious diseases*, 23, 332–9.

Gaynes R and Edwards JR (2005) Overview of nosocomial infections caused by gram-negative bacilli. *Clinical infectious diseases*, 41, 848–54.

Giedraitienė A, Vitkauskienė A, Naginienė R, *et al*. (2011) Antibiotic resistance mechanisms of clinically important bacteria. *Medicina (Kaunas, Lithuania)*, 47, 137–46.

Gillings MR (2013) Evolutionary consequences of antibiotic use for the resistome, mobilome and microbial pangenome. *Frontiers in microbiology*, 4, 4.

González G, Ramírez C and Muñoz M (2000) [Antibacterial activity of aminoglycosides against nosocomial strains of *Acinetobacter baumannii* isolated between 1990 and 1999]. *Revista española de quimioterapia*, 13, 401–4.

Griffin MO, Fricovsky E, Ceballos G, *et al*. (2010) Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature. *American journal of physiology*, 299, C539–48.

Guardabassi L, Dijkshoorn L, Collard JM, *et al*. (2000) Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *Journal of medical microbiology*, 49, 929–36.

Guay DR and Koskoletos C (1985) Aztreonam, a new monobactam antimicrobial. *Clinical pharmacy*, 4, 516–26.

Guilfoile PG (2007) *Antiobitic-resistant Bacteria*. New York: Infobase Publishing.

Gunderson BW, Ibrahim KH, Hovde LB, *et al*. (2003) Synergistic activity of colistin and ceftazidime against multiantibiotic-resistant *Pseudomonas aeruginosa* in an in vitro pharmacodynamic model. *Antimicrobial agents and chemotherapy*, 47, 905–9.

Gundi VAKB, Dijkshoorn L, Burignat S, *et al*. (2009) Validation of partial *rpoB* gene sequence analysis for the identification of clinically important and emerging *Acinetobacter* species. *Microbiology*, 155, 2333–41.

Hacker J and Kaper JB (2000) Pathogenicity islands and the evolution of microbes. *Annual review of microbiology*, 54, 641–79.

Hall RM (2012) Integrons and gene cassettes: hotspots of diversity in bacterial genomes. *Annals of the New York Academy of Sciences*, 1267, 71–8.

Hall RM and Collis CM (1995) Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular microbiology*, 15, 593–600.

Hall RM and Collis CM (1998) Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug resistance updates,* 1, 109–19.

Hamouda A, Evans BA, Towner KJ, *et al*. (2010) Characterization of epidemiologically unrelated *Acinetobacter baumannii* isolates from four continents by use of multilocus sequence typing, pulsed-field gel electrophoresis, and sequence-based typing of bla(OXA-51-like) genes. *Journal of clinical microbiology*, 48, 2476–83.

Hawkey PM (1998) The origins and molecular basis of antibiotic resistance. *BMJ*, 317, 657–60.

Heep M, Brandstätter B, Rieger U, *et al*. (2001) Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *Journal of clinical microbiology*, 39, 107–10.

Héritier C, Poirel L, Fournier P-E, *et al*. (2005) Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 49, 4174–9.

Héritier C, Poirel L, Lambert T, *et al*. (2005) Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 49, 3198–202.

Héritier C, Poirel L and Nordmann P (2006) Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*. *Clinical microbiology and infection*, 12, 123–30.

Higgins PG, Wisplinghoff H, Stefanik D, *et al*. (2004) Selection of topoisomerase mutations and overexpression of *adeB* mRNA transcripts during an outbreak of *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 54, 821–3.

Higgins PG, Poirel L, Lehmann M, *et al*. (2009) OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 53, 5035–8.

Higgins PG, Dammhayn C, Hackel M, *et al*. (2010) Global spread of carbapenem-resistant *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 65, 233–8.

Higgins PG, Pérez-Llarena FJ, Zander E, *et al*. (2013) OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 57, 2121–6.

Hiramatsu K, Cui L, Kuroda M, *et al*. (2001) The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends in microbiology*, 9, 486–93.

Ho JM-W and Juurlink DN (2011) Considerations when prescribing trimethoprim-sulfamethoxazole. *Canadian Medical Association journal,* 183, 1851–8.

Hooper DC (1999) Mode of action of fluoroquinolones. *Drugs*, 58 Suppl 2, 6–10.

Houang ETS, Chu Y-W, Lo W-S, *et al*. (2003) Epidemiology of Rifampin ADP-Ribosyltransferase (*arr-2*) and Metallo-β-Lactamase ($bla_{\text{IMP-4}}$) Gene Cassettes in Class 1 Integrons in *Acinetobacter* Strains Isolated from Blood Cultures in 1997 to 2000. *Antimicrobial Agents and Chemotherapy*, 47, 1382–1390.

Howard A, O'Donoghue M, Feeney A, *et al*. (2012) *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence*, 3, 243–50.

Hughes D (2003) Exploiting genomics, genetics and chemistry to combat antibiotic resistance. *Nature reviews. Genetics*, 4, 432–41.

Hujer KM, Hamza NS, Hujer AM, *et al*. (2005) Identification of a new allelic variant of the *Acinetobacter baumannii* cephalosporinase, ADC-7 beta-lactamase: defining a unique family of class C enzymes. *Antimicrobial agents and chemotherapy*, 49, 2941–8.

Huovinen P (2001) Resistance to trimethoprim-sulfamethoxazole. *Clinical infectious diseases,* 32, 1608–14.

Huovinen P, Sundström L, Swedberg G, *et al*. (1995) Trimethoprim and sulfonamide resistance. *Antimicrobial agents and chemotherapy*, 39, 279–89.

Iacono M, Villa L, Fortini D, *et al*. (2008) Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. *Antimicrobial agents and chemotherapy*, 52, 2616–25.

Ibrahim A, Gerner-Smidt P and Liesack W (1997) Phylogenetic relationship of the twenty-one DNA groups of the genus *Acinetobacter* as revealed by 16S ribosomal DNA sequence analysis. *International journal of systematic bacteriology*, 47, 837–41.

Jankowski S, Grzybek-Hryncewicz K, Fleischer M, *et al*. (1992) Susceptibility of isolates of *Acinetobacter anitratus* and *Acinetobacter lwoffii* to the bactericidal activity of normal human serum. *FEMS microbiology immunology*, 4, 255–60.

Jiménez-Mejías ME, Pichardo-Guerrero C, Márquez-Rivas FJ, *et al*. (2002) Cerebrospinal fluid penetration and pharmacokinetic/pharmacodynamic parameters of intravenously administered colistin in a case of multidrug-resistant *Acinetobacter baumannii* meningitis. *European journal of clinical microbiology & infectious diseases*, 21, 212–4.

Johnson DH and Cunha BA (1995) Aztreonam. *The Medical clinics of North America*, 79, 733–43.

Jones RN, Ferraro MJ, Reller LB, *et al*. (2007) Multicenter studies of tigecycline disk diffusion susceptibility results for *Acinetobacter* spp. *Journal of clinical microbiology*, 45, 227–30.

Juhas M, van der Meer JR, Gaillard M, *et al*. (2009) Genomic islands: tools of bacterial horizontal gene transfer and evolution. *FEMS microbiology reviews*, 33, 376–93.

Kaase M, Nordmann P, Wichelhaus TA, *et al*. (2011) NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. *The Journal of antimicrobial chemotherapy*, 66, 1260–2.

Kahan JS, Kahan FM, Goegelman R, *et al*. (1979) Thienamycin, a new beta-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. *The Journal of antibiotics*, 32, 1–12.

Kahne D, Leimkuhler C, Lu W, *et al*. (2005) Glycopeptide and lipoglycopeptide antibiotics. *Chemical reviews*, 105, 425–48.

Kalman D and Barriere SL (1990) Review of the pharmacology, pharmacokinetics, and clinical use of cephalosporins. *Texas Heart Institute journal*, 17, 203–15.

Kashiwagi K, Miyaji A, Ikeda S, *et al*. (1992) Increase of sensitivity to aminoglycoside antibiotics by polyamine-induced protein (oligopeptide-binding protein) in *Escherichia coli*. *Journal of bacteriology*, 174, 4331–7.

Keen EF, Robinson BJ, Hospenthal DR, *et al*. (2010) Prevalence of multidrug-resistant organisms recovered at a military burn center. *Burns*, 36, 819–25.

Kim C-K, Lee Y, Lee H, *et al*. (2010) Prevalence and diversity of carbapenemases among imipenem-nonsusceptible *Acinetobacter* isolates in Korea: emergence of a novel OXA-182. *Diagnostic microbiology and infectious disease*, 68, 432–8.

Kisgen J and Whitney D (2008) Ceftobiprole, a Broad-Spectrum Cephalosporin With Activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Pharmacy & Therapeutics,* 33, 631–41.

Knapp K and English K (2001) Carbapenems. *Seminars in Pediatric Infectious Diseases*, 12, 175–185.

Kollef M (2009) New antimicrobial agents for methicillin- resistant *Staphylococcus aureus*. *Critical care and resuscitation*, 11, 282–286.

Krizova L and Nemec A (2010) A 63 kb genomic resistance island found in a multidrug-resistant *Acinetobacter baumannii* isolate of European clone I from 1977. *The Journal of antimicrobial chemotherapy*, 65, 1915–8.

Kwa AL, Tam VH and Falagas ME (2008) Polymyxins: a review of the current status including recent developments. *Annals of the Academy of Medicine*, 37, 870–83.

Landman D, Georgescu C, Martin DA, *et al*. (2008) Polymyxins revisited. *Clinical microbiology reviews*, 21(3), 449–65.

Langille MGI, Hsiao WWL and Brinkman FSL (2010) Detecting genomic islands using bioinformatics approaches. *Nature reviews. Microbiology*, 8, 373–82.

Lee K, Yum JH, Yong D, *et al*. (2005) Novel acquired metallo-beta-lactamase gene, $bla_{\text{SIM-1}}$, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrobial agents and chemotherapy*, 49, 4485–91.

Lee Y, Kim C-K, Lee H, *et al*. (2011) A novel insertion sequence, IS*Aba10*, inserted into IS*Aba1* adjacent to the bla(OXA-23) gene and disrupting the outer membrane protein gene *carO* in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 55, 361–3.

Lee Y, Bae IK, Kim J, *et al*. (2012) Dissemination of ceftazidime-resistant *Acinetobacter baumannii* clonal complex 92 in Korea. *Journal of applied microbiology*, 112, 1207–11.

Leung W-S, Chu C-M, Tsang K-Y, *et al*. (2006) Fulminant community-acquired *Acinetobacter baumannii* pneumonia as a distinct clinical syndrome. *Chest*, 129, 102–9.

Ligon BL (2004) Penicillin: its discovery and early development. *Seminars in pediatric infectious diseases*, 15, 52–7.

Lin L, Ling B-D and Li X-Z (2009) Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter* calcoaceticus complex. *International journal of antimicrobial agents*, 33, 27–32.

Livermore DM, Sefton AM and Scott GM (2003) Properties and potential of ertapenem. *The Journal of antimicrobial chemotherapy*, 52, 331–44.

Lopes BS, Gould IM, Opazo AF, *et al*. (2012a) The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary. *International journal of antimicrobial agents*, 39, 361–2.

Lopes BS, Al-Hassan L and Amyes SGB (2012b) IS*Aba825* controls the expression of the chromosomal bla(OXA-51-like) and the plasmid borne bla(OXA-58) gene in clinical isolates of *Acinetobacter baumannii* isolated from the USA. *Clinical microbiology and infection*, 18, 446–51.

Lopes BS, Gallego L and Amyes SGB (2013) Multi-drug resistance profiles and the genetic features of *Acinetobacter baumannii* isolates from Bolivia. *Journal of infection in developing countries*, 7, 323–8.

Lu P-L, Doumith M, Livermore DM, *et al*. (2009) Diversity of carbapenem resistance mechanisms in *Acinetobacter baumannii* from a Taiwan hospital: spread of plasmid-borne OXA-72 carbapenemase. *The Journal of antimicrobial chemotherapy*, 63, 641–7.

Luna CM and Aruj PK (2007) Nosocomial *Acinetobacter* pneumonia. *Respirology*, 12, 787–91.

Mahillon J and Chandler M (1998) Insertion sequences. *Microbiology and molecular biology reviews,* 62, 725–74.

Mak JK, Kim M-J, Pham J, *et al*. (2009) Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 63, 47–54.

Malone L and Kwon DH (2013) Carbapenem-associated multidrug-resistant *Acinetobacter baumannii* are sensitised by aztreonam in combination with polyamines. *International journal of antimicrobial agents*, 41, 70–4.

Manyando C, Njunju EM, D'Alessandro U, *et al*. (2013) Safety and efficacy of co-trimoxazole for treatment and prevention of *Plasmodium falciparum* malaria: a systematic review. *PloS one*, 8, e56916.

Martinez JL and Baquero F (2000) Mutation Frequencies and Antibiotic Resistance. *Antimicrobial Agents and Chemotherapy*, 44, 1771–1777.

Martínez P and Mattar S (2012) Imipenem-resistant *Acinetobacter baumannii* carrying the IS*Aba1-bla*$_{\text{OXA-23,51}}$ and IS*Aba1-bla*$_{\text{ADC-7}}$ genes in Monteria, Colombia. *Brazilian journal of microbiology,* 43, 1274–80.

Massova I and Mobashery S (1998) Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrobial agents and chemotherapy*, 42, 1–17.

McCammon MT, Gillette JS, Thomas DP, *et al*. (2005) Detection of *rpoB* mutations associated with rifampin resistance in *Mycobacterium tuberculosis* using denaturing gradient gel electrophoresis. *Antimicrobial agents and chemotherapy*, 49, 2200–9.

McConnell MJ, Actis L and Pachón J (2013) *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS microbiology reviews*, 37, 130–55.

McCullough JL and Maren TH (1973) Inhibition of dihydropteroate synthetase from *Escherichia coli* by sulfones and sulfonamides. *Antimicrobial agents and chemotherapy*, 3, 665–9.

Merkier AK, Catalano M, Ramírez MS, *et al*. (2008) Polyclonal spread of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ in *Acinetobacter baumannii* isolates from Argentina. *Journal of infection in developing countries*, 2, 235–40.

Metan G, Alp E, Aygen B, *et al*. (2007) *Acinetobacter baumannii* meningitis in post-neurosurgical patients: clinical outcome and impact of carbapenem resistance. *The Journal of antimicrobial chemotherapy*, 60, 197–9.

Miller EL (2002) The penicillins: a review and update. *Journal of midwifery & women's health*, 47, 426–34.

Mingeot-Leclercq MP, Glupczynski Y and Tulkens PM (1999) Aminoglycosides: activity and resistance. *Antimicrobial agents and chemotherapy*, 43, 727–37.

Miriagou V, Carattoli A and Fanning S (2006) Antimicrobial resistance islands: resistance gene clusters in *Salmonella* chromosome and plasmids. *Microbes and Infection*, 8, 1923–1930.

Mishra S, Sen MR, Upadhyay S, *et al*. (2013) Genetic linkage of *bla*$_{\text{NDM}}$ among nosocomial isolates of *Acinetobacter baumannii* from a tertiary referral hospital in northern India. *International journal of antimicrobial agents*, 41, 452–6.

Moazed D and Noller HF (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, 327, 389–94.

Moellering RC, Eliopoulos GM and Sentochnik DE (1989) The carbapenems: new broad spectrum beta-lactam antibiotics. *The Journal of antimicrobial chemotherapy*, 24 Suppl A, 1–7.

Moffatt JH, Harper M, Harrison P, *et al*. (2010) Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial agents and chemotherapy*, 54, 4971–7.

Morgan AA and Rubenstein E (2013) Proline: the distribution, frequency, positioning, and common functional roles of proline and polyproline sequences in the human proteome., *PloS one*, 8, e53785.

Moskowitz SM, Ernst RK and Miller SI (2004) PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *Journal of bacteriology*, 186, 575–9.

Mugnier PD, Poirel L, Naas T, *et al*. (2010) Worldwide dissemination of the $bla_{\text{OXA-23}}$ carbapenemase gene of *Acinetobacter baumannii*. *Emerging infectious diseases*, 16, 35–40.

Mussi MA, Relling VM, Limansky AS, *et al*. (2007) CarO, an *Acinetobacter baumannii* outer membrane protein involved in carbapenem resistance, is essential for L-ornithine uptake. *FEBS letters*, 581, 5573–8.

Naas T, Namdari F, Réglier-Poupet H, *et al*. (2007) Panresistant extended-spectrum beta-lactamase SHV-5-producing *Acinetobacter baumannii* from New York City. *The Journal of antimicrobial chemotherapy*, 60, 1174–6.

Naber KG and Adam D (1998) Classification of fluoroquinolones. *International journal of antimicrobial agents*, 10, 255–7.

Nakamatsu EH, Fujihira E, Ferreira RCC, *et al*. (2007) Oligopeptide uptake and aminoglycoside resistance in *Escherichia coli* K12. *FEMS microbiology letters*, 269, 229–33.

Nakazawa Y, Ii R, Tamura T, *et al*. (2013) A case of NDM-1-producing *Acinetobacter baumannii* transferred from India to Japan. *Journal of infection and chemotherapy*, 19, 330–2.

Navon-Venezia S, Leavitt A and Carmeli Y (2007) High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 59, 772–4.

Nelson ML and Levy SB (2011) The history of the tetracyclines. *Annals of the New York Academy of Sciences*, 1241, 17–32.

Nemec A, Maixnerová M, van der Reijden TJK, *et al*. (2007) Relationship between the AdeABC efflux system gene content, netilmicin susceptibility and multidrug resistance in a genotypically diverse collection of *Acinetobacter baumannii* strains. *The Journal of antimicrobial chemotherapy*, 60, 483–9.

Nemec A, Musílek M, Maixnerová M, *et al*. (2009) *Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov., haemolytic organisms isolated from humans. *International journal of systematic and evolutionary microbiology*, 59, 118–24.

Nemec A, Musílek M, Sedo O, *et al*. (2010) *Acinetobacter bereziniae* sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. *International journal of systematic and evolutionary microbiology*, 60, 896–903.

Nemec A, Krizova L, Maixnerova M, *et al*. (2011) Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU). *Research in microbiology*, 162, 393–404.

Nicolau DP (2008) Carbapenems: a potent class of antibiotics. *Expert opinion on pharmacotherapy*, 9, 23–37.

Nicolosi D, Scalia M, Nicolosi VM, *et al*. (2010) Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. *International journal of antimicrobial agents*, 35, 553–8.

Nikaido H (2009) Multidrug resistance in bacteria. *Annual review of biochemistry*, 78, 119–46.

Norrby SR (1995) Carbapenems. *The Medical clinics of North America*, 79, 745–59.

Noskin GA (2005) Tigecycline: a new glycylcycline for treatment of serious infections. *Clinical infectious diseases*, 41 Suppl 5, S303–14.

Okeke IN, Klugman KP, Bhutta ZA, *et al*. (2005) Antimicrobial resistance in developing countries. Part II: strategies for containment. *The Lancet infectious diseases*, 5, 568–80.

Oliphant CM and Green GM (2002) Quinolones: a comprehensive review. *American family physician*, 65, 455–64.

Olson MW, Ruzin A, Feyfant E, *et al*. (2006) Functional, biophysical, and structural bases for antibacterial activity of tigecycline. *Antimicrobial agents and chemotherapy*, 50, 2156–66.

Olut AI and Erkek E (2005) Early prosthetic valve endocarditis due to *Acinetobacter baumannii*: a case report and brief review of the literature. *Scandinavian journal of infectious diseases*, 37, 919–21.

Opazo Andres, Sonnevend A, Lopes B, *et al*. (2012a) Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates. *The Journal of antimicrobial chemotherapy*, 67, 1619–22.

Opazo Andrés, Domínguez M, Bello H, *et al*. (2012b) OXA-type carbapenemases in *Acinetobacter baumannii* in South America. *Journal of infection in developing countries*, 6, 311–6.

Opazo A, Vali L, Al Obaid K, *et al*. (2014) Novel genetic structure harbouring $bla_{\text{PER-1}}$ in ceftazidime-resistant *Acinetobacter baumannii* isolated from Kuwait. *International journal of antimicrobial agents*, 43, 383-4.

Pachón-Ibáñez ME, Fernández-Cuenca F, Docobo-Pérez F, *et al*. (2006) Prevention of rifampicin resistance in *Acinetobacter baumannii* in an experimental pneumonia murine model, using rifampicin associated with imipenem or sulbactam. *The Journal of antimicrobial chemotherapy*, 58, 689–92.

Pachón-Ibáñez ME, Docobo-Pérez F, López-Rojas R, *et al*. (2010) Efficacy of rifampin and its combinations with imipenem, sulbactam, and colistin in experimental models of infection caused by imipenem-resistant *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 54, 1165–72.

Palzkill T (2013) Metallo-β-lactamase structure and function. *Annals of the New York Academy of Sciences*, 1277, 91–104.

Pan XS and Fisher LM (1997) Targeting of DNA gyrase in *Streptococcus pneumoniae* by sparfloxacin: selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrobial agents and chemotherapy*, 41, 471–4.

Partridge SR, Tsafnat G, Coiera E, *et al*. (2009) Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS microbiology reviews*, 33, 757–84.

Paton R, Miles RS, Hood J, *et al*. (1993) ARI 1: beta-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. *International journal of antimicrobial agents*, 2, 81–7.

Peleg AY, Seifert H and Paterson DL (2008) *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical microbiology reviews*, 21, 538–82.

Poirel L, Cabanne L, Vahaboglu H, *et al*. (2005a) Genetic environment and expression of the extended-spectrum beta-lactamase $bla_{\text{PER-1}}$ gene in gram-negative bacteria. *Antimicrobial agents and chemotherapy*, 49, 1708–13.

Poirel L, Marqué S, Héritier C, *et al*. (2005b) OXA-58, a novel class D {beta}-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 49, 202–8.

Poirel L and Nordmann P (2006) Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene $bla_{\text{OXA-58}}$ in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 50, 1442–8.

Poirel L, Decousser J-W and Nordmann P (2003) Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a bla(CTX-M) beta-lactamase gene. *Antimicrobial agents and chemotherapy*, 47, 2938–45.

Poirel L, Naas T and Nordmann P (2008) Genetic support of extended-spectrum beta-lactamases. *Clinical microbiology and infection*, 14 Suppl 1, 75–81.

Poirel L, Bonnin RA and Nordmann P (2011) Genetic basis of antibiotic resistance in pathogenic *Acinetobacter* species. *IUBMB life*, 63, 1061–7.

Pournaras S, Markogiannakis A, Ikonomidis A, *et al*. (2006) Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58

carbapenemase in an intensive care unit. *The Journal of antimicrobial chemotherapy*, 57, 557–61.

Pournaras S, Gogou V, Giannouli M, *et al*. (2014) Single-locus-sequence-based typing of *bla*$_{\text{OXA-51-like}}$ genes for rapid assignment of *Acinetobacter baumannii* clinical isolates to international clonal lineages. *Journal of clinical microbiology*, 52, 1653–7.

Rafei R, Dabboussi F, Hamze M, *et al*. (2014) First report of *bla*$_{\text{NDM-1}}$-producing *Acinetobacter baumannii* isolated in Lebanon from civilians wounded during the Syrian war. *International journal of infectious diseases*, 21, 21–3.

Rai J, Randhawa GK and Kaur M (2013) Recent advances in antibacterial drugs. *International journal of applied & basic medical research*, 3, 3–10.

Raju TN (1999) The Nobel chronicles. 1952: Selman Abraham Waksman (1888-1973). *Lancet*, 353, 1536.

Ravasi P, Limansky AS, Rodriguez RE, *et al*. (2011) IS*Aba825*, a functional insertion sequence modulating genomic plasticity and bla(OXA-58) expression in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 55, 917–20.

Reynolds PE and Courvalin P (2005) Vancomycin resistance in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine. *Antimicrobial agents and chemotherapy*, 49, 21–5.

Reznikoff WS (2003) Tn*5* as a model for understanding DNA transposition. *Molecular microbiology*, 47, 1199–206.

Ribera A, Roca I, Ruiz J, *et al*. (2003) Partial characterization of a transposon containing the *tet*(A) determinant in a clinical isolate of *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 52, 477–80.

Robledo IE, Aquino EE, Santé MI, *et al*. (2010) Detection of KPC in *Acinetobacter* spp. in Puerto Rico. *Antimicrobial agents and chemotherapy*, 54, 1354–7.

Rodríguez-Martínez J-M, Nordmann P, Ronco E, *et al*. (2010) Extended-spectrum cephalosporinase in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 54, 3484–8.

Ruiz M, Marti S, Fernandez-Cuenca F, *et al*. (2007) Prevalence of IS*(Aba1)* in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. *FEMS microbiology letters*, 274, 63–6.

Rumbo C, Gato E, López M, *et al*. (2013) The Contribution of Efflux Pumps, Porins and β-Lactamases to Multi-drug Resistance in Clinical Isolates of *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 57, 5247-57.

Ruzin A, Keeney D and Bradford PA (2007) AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter* calcoaceticus-*Acinetobacter baumannii* complex. *The Journal of antimicrobial chemotherapy*, 59, 1001–4.

Schindler M and Osborn MJ (1979) Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochemistry*, 18, 4425–30.

Schlünzen F, Zarivach R, Harms J, *et al*. (2001) Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature*, 413, 814–21.

Schmidt H and Hensel M (2004) Pathogenicity islands in bacterial pathogenesis. *Clinical microbiology reviews*, 17, 14–56.

Sebeny PJ, Riddle MS and Petersen K (2008) *Acinetobacter baumannii* skin and soft-tissue infection associated with war trauma. *Clinical infectious diseases*, 47, 444–9.

Segal H, Thomas R and Gay Elisha B (2003) Characterization of class 1 integron resistance gene cassettes and the identification of a novel IS-like element in *Acinetobacter baumannii*. *Plasmid*, 49, 169–78.

Segal H, Garny S and Elisha BG (2005) Is IS(ABA-1) customized for *Acinetobacter*? *FEMS microbiology letters*, 243, 425–9.

Seifert H, Strate A, Schulze A, *et al*. (1994) Bacteremia due to *Acinetobacter* species other than *Acinetobacter baumannii*. *Infection*, 22, 379–85.

Seifert H, Dolzani L, Bressan R, *et al*. (2005) Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *Journal of clinical microbiology*, 43, 4328–35.

Sevillano E, Fernández E, Bustamante Z, *et al*. (2012) Emergence and clonal dissemination of carbapenem-hydrolysing OXA-58-producing *Acinetobacter baumannii* isolates in Bolivia. *Journal of medical microbiology*, 61, 80–4.

Shah F (1998) Erythromycin. *Pediatrics in review*, 19, 140–1.

Shlaes DM (2010) *Antibiotics: The Perfect Storm*. New York: Springer.

Siefert JL (2009) Defining the mobilome. *Methods in molecular biology*, 532, 13–27.

Silva O F, Cifuentes D M and Pinto C ME (2011) Resultados de la vigilancia de susceptibilidad antimicrobiana en Chile: Consolidando una red. *Revista chilena de infectología*, Sociedad Chilena de Infectología, 28, 19–27.

Sköld O (2000) Sulfonamide resistance: mechanisms and trends. *Drug resistance updates*, 3, 155–160.

Sköld O (2001) Resistance to trimethoprim and sulfonamides. *Veterinary research*, EDP Sciences, 32, 261–73.

Sköld O (2011) *Antibiotics and Antibiotic Resistance*. New Jersey: Wiley.

Song MD, Wachi M, Doi M, *et al*. (1987) Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. *FEBS letters*, 221, 167–71.

Spence RP and Towner KJ (2003) Frequencies and mechanisms of resistance to moxifloxacin in nosocomial isolates of *Acinetobacter baumannii*. *The Journal of antimicrobial chemotherapy*, 52, 687–90.

Stein GE and Babinchak T (2013) Tigecycline: an update. *Diagnostic microbiology and infectious disease*, 75, 331–6.

Stein GE and Craig WA (2006) Tigecycline: a critical analysis. *Clinical infectious diseases*, 43, 518–24.

Stokes HW and Hall RM (1989) A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Molecular microbiology*, 3, 1669–83.

Strevel EL, Kuper A and Gold WL (2006) Severe and protracted hypoglycaemia associated with co-trimoxazole use. *The Lancet infectious diseases*, 6, 178–82.

Su X-Z, Chen J, Mizushima T, *et al*. (2005) AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrobial agents and chemotherapy*, 49, 4362–4.

Taber HW, Mueller JP, Miller PF, *et al*. (1987) Bacterial uptake of aminoglycoside antibiotics. *Microbiological reviews*, 51, 439–57.

Tascini C, Ferranti S, Messina F, *et al*. (2000) In vitro and in vivo synergistic activity of colistin, rifampin, and amikacin against a multiresistant *Pseudomonas aeruginosa* isolates. *Clinical microbiology and infection*, 6, 690–1.

Tavakoli N, Comanducci A, Dodd HM, *et al*. (2000) IS*1294*, a DNA element that transposes by RC transposition. *Plasmid*, 44, 66–84.

Telenti A, Imboden P, Marchesi F, *et al*. (1993) Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *The Lancet*, 341, 647–651.

Thapa B, Tribuddharat C, Rugdeekha S, *et al*. (2009) Rifampin resistance in carbapenem-resistant *Acinetobacter baumannii* in Siriraj Hospital, Thailand. *Nepal Medical College journal*, 11, 232–7.

Tian G-B, Adams-Haduch JM, Taracila M, *et al*. (2011) Extended-spectrum AmpC cephalosporinase in *Acinetobacter baumannii*: ADC-56 confers resistance to cefepime. *Antimicrobial agents and chemotherapy*, 55, 4922–5.

Tognim MCB, Andrade SS, Silbert S, *et al*. (2004) Resistance trends of *Acinetobacter* spp. in Latin America and characterization of international dissemination of multi-drug resistant strains: five-year report of the SENTRY Antimicrobial Surveillance Program. *International journal of infectious diseases*, 8, 284–91.

Toleman MA, Bennett PM and Walsh TR (2006) IS*CR* elements: novel gene-capturing systems of the 21st century? *Microbiology and molecular biology reviews*, 70, 296–316.

Toleman MA, Spencer J, Jones L, *et al*. (2012) *bla*$_{NDM-1}$ is a chimera likely constructed in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*, 56, 2773–6.

Toussaint A and Chandler M (2012) Prokaryote genome fluidity: toward a system approach of the mobilome. *Methods in molecular biology*, 804, 57–80.

Towner KJ (2009) *Acinetobacter*: an old friend, but a new enemy. *The Journal of hospital infection*, 73, 355–63.

Tribuddharat C and Fennewald M (1999) Integron-mediated rifampin resistance in *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy*, 43, 960–2.

Tritton TR (1977) Ribosome-tetracycline interactions. *Biochemistry*, American Chemical Society, 16, 4133–4138.

Tsakris A, Ikonomidis A, Pournaras S, *et al*. (2006) VIM-1 metallo-beta-lactamase in *Acinetobacter baumannii*. *Emerging infectious diseases*, 12, 981–3.

Turton JF, Ward ME, Woodford N, *et al*. (2006a) The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS microbiology letters*, 258, 72–7.

Turton JF, Woodford N, Glover J, *et al*. (2006b) Identification of *Acinetobacter baumannii* by detection of the *bla*$_{OXA-51-like}$ carbapenemase gene intrinsic to this species. *Journal of clinical microbiology*, 44, 2974–6.

Turton JF, Gabriel SN, Valderrey C, *et al*. (2007) Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clinical microbiology and infection*, 13, 807–15.

Vaara M (2010) Polymyxins and their novel derivatives. *Current opinion in microbiology*, 13, 574–81.

Vahaboglu H, Budak F, Kasap M, *et al*. (2006) High prevalence of OXA-51-type class D beta-lactamases among ceftazidime-resistant clinical isolates of *Acinetobacter* spp.: co-existence with OXA-58 in multiple centres. *The Journal of antimicrobial chemotherapy*, 58, 537–42.

Van Bambeke F (2006) Glycopeptides and glycodepsipeptides in clinical development: a comparative review of their antibacterial spectrum, pharmacokinetics and clinical efficacy. *Current opinion in investigational drugs,* 7, 740–9.

Van Bambeke F, Balzi E and Tulkens PM (2000) Antibiotic efflux pumps. *Biochemical pharmacology*, 60, 457–70.

Van der Meer JR, Ravatn R and Sentchilo V (2001) The *clc* element of *Pseudomonas* sp. strain B13 and other mobile degradative elements employing phage-like integrases. *Archives of microbiology*, 175, 79–85.

Van Niekerk CH, Steyn DL, Davis WG, *et al*. (1980) Chloramphenicol levels in cerebrospinal fluid in meningitis. *South African medical journal*, 58, 159–60.

Walsh C (2003) *Antibiotics. Actions, origins, resistance*. Washington, DC: ASM Press.

Walsh TR, Toleman MA, Poirel L, *et al*. (2005) Metallo-beta-lactamases: the quiet before the storm? *Clinical microbiology reviews*, 18, 306–25.

Wareham DW and Wilson P (2002) Chloramphenicol in the 21st century. *Hospital medicine*, 63, 157–61.

Weinbauer MG and Rassoulzadegan F (2004) Are viruses driving microbial diversification and diversity? *Environmental microbiology*, 6, 1–11.

Weinstein MJ, Luedemann GM, Oden EM, *et al*. (1963) Gentamicin, a new antibiotic complex from *Micromonospora*. *Journal of medicinal chemistry*, 6, 463–4.

Wisplinghoff H, Bischoff T, Tallent SM, *et al*. (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical infectious diseases*, 39, 309–17.

Woodford N, Ellington MJ, Coelho JM, *et al*. (2006) Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *International journal of antimicrobial agents*, 27, 351–3.

Wright AJ and Wilkowske CJ (1987) The penicillins. *Mayo Clinic proceedings. Mayo Clinic*, 62, 806–20.

Wright GD (1999) Aminoglycoside-modifying enzymes. *Current opinion in microbiology*, 2, 499–503.

Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature reviews. Microbiology*, 5, 175–86.

Xia R, Guo X, Zhang Y, *et al*. (2010) *qnrVC*-like gene located in a novel complex class 1 integron harboring the IS*CR1* element in an *Aeromonas punctata* strain from an aquatic environment in Shandong Province, China. *Antimicrobial agents and chemotherapy*, 54, 3471–4.

Yamamoto T (1989) Organization of complex transposon Tn*2610* carrying two copies of *tnpA* and *tnpR*. *Antimicrobial agents and chemotherapy*, 33, 746–50.

Yamamoto T, Watanabe M, Matsumoto K, *et al*. (1983) Tn*2610*, a transposon involved in the spread of the carbenicillin-hydrolyzing beta-lactamase gene. *Molecular & general genetics*, 189, 282–8.

Yu Y, Zhou H, Yang Q, *et al*. (2007) Widespread occurrence of aminoglycoside resistance due to ArmA methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *The Journal of antimicrobial chemotherapy*, 60, 454–5.

Zander E, Nemec A, Seifert H, *et al*. (2012) Association between β-lactamase-encoding bla(OXA-51) variants and DiversiLab rep-PCR-based typing of *Acinetobacter baumannii* isolates. *Journal of clinical microbiology*, 50, 1900–4.

Zander E, Higgins PG, Fernández-González A, *et al*. (2013) Detection of intrinsic *bla*$_{\text{OXA-51-like}}$ by multiplex PCR on its own is not reliable for the identification of *Acinetobacter baumannii*. *International journal of medical microbiology*, 303, 88–9.

Zarrilli R, Pournaras S, Giannouli M, *et al*. (2013) Global evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages. *International journal of antimicrobial agents*, 41, 11–9.

Zhanel GG, Dueck M, Hoban DJ, *et al*. (2001) Review of macrolides and ketolides: focus on respiratory tract infections. *Drugs*, 61, 443–98.

Zhanel GG, Johanson C, Embil JM, *et al*. (2005) Ertapenem: review of a new carbapenem. *Expert review of anti-infective therapy*, 3, 23–39.

Zhanel GG, Wiebe R, Dilay L, *et al*. (2007) Comparative review of the carbapenems. *Drugs*, 67, 1027–52.

Zowawi HM, Balkhy HH, Walsh TR, *et al*. (2013) β-Lactamase production in key gram-negative pathogen isolates from the Arabian Peninsula. *Clinical microbiology reviews*, 26, 361–80.

APPENDIX A:

Published papers

Review Article

OXA-type carbapenemases in *Acinetobacter baumannii* in South America

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Abstract

Acinetobacter baumannii is an opportunistic pathogen that is frequently involved in outbreaks of infection, occurring mostly in intensive care units. The increasing incidence of carbapenem resistance in *A. baumannii* worldwide is a concern since it limits drastically the range of therapeutic alternatives. The most important mechanism of carbapenem resistance is the enzymatic hydrolysis mediated by carbapenemases. In *A. baumannii* these enzymes are usually OXA-type carbapenemases, and belong to class D according to the classification of Ambler. The OXA-type carbapenemases are divided into five subgroups, four of which correspond to acquired carbapenemases, which accounts for the distribution of genes bla_{OXA} in different geographic areas. In this work we review the different types of OXA-type carbapenemases present in *A. baumannii*, emphasizing the current situation in South America with special mention to the findings in Chile.

Key words: *Acinetobacter baumannii*, carbapenem-resistance, OXA-type carbapenemases, South America, Chile

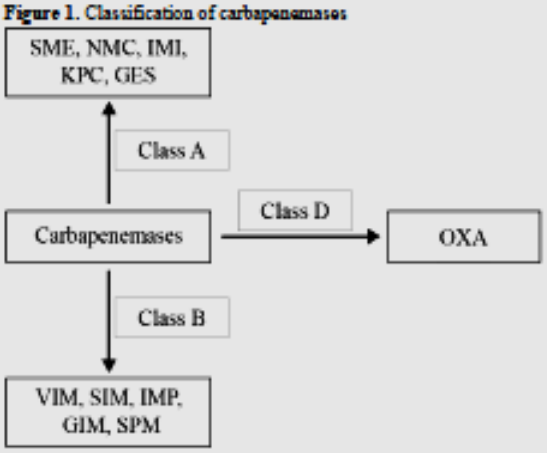
J Infect Dev Ctries 2012, 6(4):311-316.

(Received 13 September 2011 – Accepted 22 December 2011)

Introduction

Acinetobacter baumannii is a glucose non-fermentative Gram-negative bacillus classified as an opportunistic pathogen and is usually involved in infectious outbreaks originating in intensive care units [1]. The infections caused by *A. baumannii* include bloodstream infections and ventilator-associated pneumonia [2]. The beta-lactam antibiotics are an important group of antibiotics used to treat infections caused by various microorganisms, including *A. baumannii*, due to their efficacy and safety, and because their activity can be increased by chemical modification [3,4]. Until the 1970s, most clinical isolates of *Acinetobacter* spp. were susceptible to most groups of antibiotics available, including beta-lactams; however, the species *A. baumannii* has a great ability to develop resistance against antibiotics [3], which has generated, over the last decade, an increase in the number of multidrug resistant (MDR) isolates of *A. baumannii* [5]. Due to the increased rate of resistance to other antibiotics, the use of carbapenem antibiotics has become necessary in *A. baumannii* infections. This class of antibiotics exhibits a broad-spectrum activity against Gram-negative and Gram-positive organisms, including anaerobic bacteria; and is therefore used to treat serious infections [4]. Carbapenems, except for ertapenem, are active against *A. baumannii* and have become the drugs of choice for infections caused by this bacterium. Carbapenems are generally more active and more resistant to beta-lactamases, including extended spectrum beta-lactamases and derepressed chromosomal AmpC beta-lactamases due mainly to the characteristics of the lateral chains present in its chemical structure [6]. The resistance to these compounds is not due to the presence of a single mechanism, but to a combination of different mechanisms classified as enzymatic and non-enzymatic with most important mechanism of resistance to this class of antibiotics the enzymatic hydrolysis, mediated by enzymes called carbapenemases [7]. These enzymes belong to any of three molecular classes according to the Ambler classification [8] (Figure 1). Class A includes beta-lactamases that possess serine in their active site and are inhibited by clavulanic acid. These carbapenemases are part of the Bush functional group 2f [9], and have been detected mainly in

Figure 1. Classification of carbapenemases



Enterobacteriaceae; however, KPC-like enzymes and GES-like enzymes have been detected in carbapenem-resistant *A. baumannii* strains isolated in Puerto Rico and Paris, respectively [10,11]. Class B includes zinc dependant beta-lactamases named metallo-beta-lactamases (MBL), which are part of the functional group 3 [7,9]. To date, the presence of IMP, VIM, and SIM-1 groups of MBL has been identified in *A. baumannii* [5,12]. Finally, the third molecular class corresponds to class D serine-carbapenemases, called OXA-type carbapenemases (OTC), which belongs to the functional group 2d [7,9]. Although OTC have a lower catalytic efficiency to hydrolyse carbapenems in comparison with MBL (100 to 1000 fold lower), it is important to consider them as potentially dangerous because their expression can be regulated by the upstream insertion of IS elements such as IS*Aba1* [13,14]. This can be intensified when other mechanisms of resistance are present, such as increased expression of efflux pumps and loss of porins [15,16,17]. The most prevalent carbapenemases in *A. baumannii* are the OXA-type-beta-lactamases [12] and, for this reason, this review analyses molecular and epidemiological aspects of OTC in *A. baumannii* emphasizing the current situation in South America, and the latest finding in Chilean strains of *A. baumannii*.

General properties of OXA-type beta-lactamases

Originally, the denomination OXA was due to the capacity of this group of carbapenemases to hydrolyze isoxazolylpenicillin oxacillin faster than classical penicillins and the fact that they are not inhibited by clavulanic acid and EDTA [7,9]. However, today this definition is not valid because since enzymes have been recently described that inactivate cloxacillin and oxacillin weakly, but all OXA beta-lactamases are active against amino- and carboxipenicillins [18]. Multiple alignment analysis of the sequences of these enzymes identifies three highly conserved active-site elements [18,19]. The first corresponds to a tetrad, composed of Ser^{70}-X-X-Lys, in which X corresponds to any amino acid, and the serine in position 70 corresponds to the amino acid of the active site. The second element corresponds to Ser^{128}-X-Val/Ile. The third element is represented by the Tyr/Phe^{144}-Gly-Asn triad plus the Trp^{212}-X-X-Gly tetrad, which have no analogues in class A or AmpC enzymes [19].

During the last four years descriptions of new OXA-type beta-lactamases have increased considerably. Thus Queenan and Bush reported in 2007 [7] that 102 unique OXA sequences had been assigned to this group, 9 of which were recognized as extended spectrum beta-lactamases and 37 as carbapenemases. Later, in 2010, Poirel *et al.*

reported 147 OXA enzymes [18], 19 of which were expanded-spectrum beta-lactamases and 64 were carbapenem-hydrolyzing class D beta-lactamases. Today this number has reached 227 OXA-type beta-lactamases [20].

OXA-type carbapenemases in *A. baumannii*

The OTC contain between 243 and 260 amino acids residues, with a molecular mass ranging from 23.0 to 35.5 kDa and pI values varying between 5.1 and 9.0 [19]. The first OTC found in *A. baumannii* was obtained from a strain isolated in 1985 in a Scottish hospital, named ARI-1 (*Acinetobacter* Resistant to Imipenem) [21] and now designated as OXA-23 [22]. The bla_{OXA-23} gene was located on a plasmid (45 kb) that was transferred to *Acinetobacter junii* [23]. However, today five main phylogenetic subgroups of OTC have been recognised in *A. baumannii*: OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like and OXA-143-like [1,24,25].

By far the largest subgroup is the OXA-51-like, which corresponds to chromosomal encoded enzymes and therefore naturally occurring OTC in *A. baumannii*. The enzymes included in this subgroup differ by 1 to 15 amino acids [19,26]. The OXA-23-like subgroup includes the following derivates in *A. baumannii*: OXA-27 and -49 [12,18], which have been identified mainly to be plasmid-mediated.

Although the OXA-23-like carbapenemase subgroup has been mainly reported in *A. baumannii*, in 2002 it was detected in a carbapenem-resistant isolate of *Proteus mirabilis* [27]. Importantly, OXA-23-like enzymes have been detected in strains of *A. radioresistens* [28], which might be the natural reservoir of these enzymes.

Enzymes belonging to OXA-58-like subgroup can be located on plasmids, which may explain their wide distribution [1]. Two variants within this subgroup have been described: OXA-96 and OXA-97 [29,30].

The fourth subgroup corresponds to the OXA-40-like, originally called OXA-24, with three variants, OXA-25, OXA-26 and OXA-72, which have been identified in plasmids [31,32]. Enzymes of this group have not been described to be associated with insertion sequences [1].

In 2009, Higgins *et al.* reported a new OTC, the OXA-143, which was described in a carbapenem-resistant *A. baumannii* strain isolated in Brazil in 2004. This enzyme was located on a *ca* 30 kb plasmid and has 88% amino acid sequence homology with OXA-40, 63% with OXA-23, and 52% with OXA-58 [24], representing a new subgroup of OTC.

The bla_{OXA} genes have been related to a variety of different genetic structures, emphasizing the insertion sequences, which have an important role in the expression of these genes, specifically IS*Aba1*, IS*Aba2*, IS*Aba3* or IS*18* in the case of *A. baumannii* [13,14,33]. Insertion sequences may result in hybrid promoter sequences associated with increased expression rates, which represents a real mechanism of resistance to carbapenems, or at least one of reduced susceptibility [13]. Many oxacillinases genes present in isolates of *A. baumannii* have been detected as gene cassettes in integrons. However, most of the OTC genes were identified in plasmids, but not in the form of genetic cassettes within integrons [19].

Epidemiology

OXA-type carbapenemases have been described around the world [25], including South America (Table 1). Strains of *A. baumannii* harbouring OXA-23-like enzymes have been identified in Brazil, Argentina and Colombia [25,34,35,36,37]. Moreover, carbapenemases belonging to OXA-23-like subgroup have been detected in Europe, Australia, Tahiti, China, Korea, Singapore, Vietnam, USA, Libya and Pakistan [1].

With respect to the subgroup OXA-58-like, which was first detected in France [33], strains of *A. baumannii* carrying bla_{OXA-58} have been identified in South America, specifically in Argentina, Colombia, Bolivia and Venezuela [25,34,35,38,39]. Additionally, a total of 38 strains of *A. baumannii* carrying the bla_{OXA-58} gene have been detected in Chile; these were isolated from three different hospitals in Santiago, Chile, between the years 2007 and 2008 [Opazo *et al.*, unpublished results, 2010]. The strains were clonally intra-hospital related, sharing more than 85% similarity among their pulsed-field gel electrophoresis (PFGE) profiles, but they were not inter-hospital related, suggesting the occurrence of a non-clonal dissemination of carbapenem-resistant *A. baumannii* strains harbouring bla_{OXA-58}.

Moreover, strains of *A. baumannii* resistant to carbapenems carrying the bla_{OXA-58} gene have been reported in European countries such as Spain, Turkey, Romania, United Kingdom, Italy, Poland, Switzerland, Germany, Ireland, Portugal, Hungary,

Table 1. Presence of OXA-type carbapenemases in South America

OTC subgroup	Countries	Genetic location	References
OXA-51	Argentina, Bolivia, Brazil, Chile*, Colombia, Venezuela	Chromosome	25, 38, 46
OXA-23	Argentina, Colombia, Brazil	Chromosome, Plasmid	25, 34, 35, 36
OXA-40	Brazil, Chile	Plasmid	42,45
OXA-58	Argentina, Bolivia, Chile*, Colombia, Bolivia, Venezuela	Plasmid	25, 35, 38, 39
OXA-143	Brazil	Plasmid	24

PBP: Penicillin binding proteins

Bulgaria, and Greece as well as in the USA, Oceania, and Asia [1,5,25]. Additionally, the bla_{OXA-58} gene was identified in strains of *A. junii* isolated in Romania and Australia [1,40] and in a clinical isolate of *Acinetobacter* genospecies 3 in Spain [41].

The OXA-40-like enzymes are the less widespread OTC [12]. In 2007, the occurrence of a plasmid OXA-40-like enzyme, OXA-72, was described in a carbapenem-resistant *A. baumannii* strain isolated in Brazil [42], that was also detected in Taiwan [32], France [43] and Croatia [44]. In Chile, two clonally unrelated strains of *A. baumannii* resistant to carbapenems isolated in 2008 were positive for OXA-40-like carbapenemases; this was the first report of the detection of this enzyme in Chile [45]. Moreover, this subgroup has been found in strains isolated in Europe (principally in Spain, Belgium, and the Czech Republic), the United States, and parts of Asia, including Iran [12].

Carbapenemases that belong to the OXA-51-like subgroup have been identified globally, due to their chromosomal location and the fact that every *A. baumannii* isolate carries an OXA-51-like gene [1]. They were first discovered in strains isolated from hospitals in Buenos Aires, Argentina, between 1993 and 1994, when the incidence of carbapenem resistance even at that time was around 35% [46]. Today, carbapenem-resistant strains of *A. baumannii* carrying an OXA-51-like enzyme have been described in various countries in South America (Table 1). Within the OXA-51-like enzymes, there are clusters of enzymes that are associated with certain epidemic lineages [26]. The cluster related to OXA-66 has been associated with an *A. baumannii* lineage including a European clone (often now called Worldwide 2), being also associated with strains from South America and Asia [26,47], whereas those clustered around OXA-69 enzyme are found in another lineage encompassing European clone 1. Finally, OXA-71 enzyme is associated with European clone 3 [26].

Conclusions

The OXA-type carbapenemases have a global distribution. Mobilization of the bla_{OXA} genes is, in some cases, determined by the presence of insertion sequences and transposons, and therefore has a high potential to spread. OXA-23-like and OXA-58-like enzymes have been detected in Brazil, Venezuela, Colombia, Chile, Bolivia and Argentina; however, OXA-58-like enzymes are most frequently identified in South America. More recently, the presence of OXA-72, from the OXA-40-like subgroup, has been reported in Brazil. This group is mostly detected in some European countries, Asia and the United States, which may indicate the spread of resistant clones from these countries to South America, and in the long term, it can mean lower effectiveness of treatment of infections caused by MDR *A. baumannii*.

Recently the first member of a novel subgroup of OXA-carbapenemases in Brazil, OXA-143, has been described and its prevalence remains to be determined. This discovery opens the possibility to find new variants in South America, which could be present in several countries of the continent. Therefore, it is important to note that the first emergences of the OXA-51, [48] and now the OXA-143 [24], as well as the second independent emergence of OXA-23 [49] carbapenemases, progenitors of three of the five types of OXA carbapenemases, were all identified in strains from South American countries in the last 20 years. It is thus very important to focus on the MDR *A. baumannii* strains from this continent, especially in countries where information is currently not available. On the other hand, the spread of bla_{OXA} genes can occur intra- and inter-hospital; therefore, it is necessary to implement rigorous control programs on infections caused by *A. baumannii* resistant to carbapenems. Furthermore, it is essential to conduct molecular genotyping studies of these strains as well as fully characterize the carbapenemases found in specific geographic areas, to prevent the spread of both genes and resistant clones.

References

1. Peleg A, Seifert H, Paterson D (2008) *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev 21: 538-582.
2. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA (2007) Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother 51: 3471-3484.
3. Livermore D and Woodford N (2006) The β-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol 14: 413-419.
4. Nicolau D (2008) Carbapenems: a potent class of antibiotics. Expert Opin Pharmacother 9: 23-37.
5. Poirel L and Nordmann P (2006) Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microbiol Infect 12: 826-836.
6. Bonfiglio G, Russo G, Nicoletti G (2002) Recent developments in carbapenems. Expert Opin Investig Drugs 11: 529-544.
7. Queenan A and Bush K (2007) Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 20: 440-458.
8. Ambler RP (1980) The structure of beta-lactamases. Philos Trans R Soc Lond B Biol Sci 289: 321-31.
9. Bush K, Jacoby G, Medeiros A (1995) A functional classification scheme for β-lactamases and its correlation to molecular structure. Antimicrob Agents Chemother 39: 1211-1233.
10. Robledo I, Aquino E, Sante M, Santana J, Otero D, Leon C, Vazquez G (2010) Detection of KPC in *Acinetobacter* spp. in Puerto Rico. Antimicrob Agents Chemother 54: 1354-1357.
11. Bonnin RA, Nordmann P, Poteon A, Lecuyer H, Zahar JR, Poirel L (2011) Carbapenem-hydrolyzing GES-type extended-spectrum beta-lactamase in *Acinetobacter baumannii*. Antimicrob Agents Chemother 55: 349-354.
12. Zarrilli R, Giannouli M, Tomasone F, Triassi M, Tsakris A (2009) Carbapenem resistance in *Acinetobacter baumannii*: the molecular epidemic features of an emerging problem in health care facilities. J Infect Dev Ctries 3: 335-341.
13. Turton J, Ward M, Woodford N, Kaufmann M, Pike R, Livermore D, Pitt T (2006) The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol Lett 258: 72-77.
14. Segal H, Garny S, Elisha BG (2005) Is IS*ABA-1* customized for *Acinetobacter*? FEMS Microbiol Lett 243: 425-429.
15. Vila J, Marti S. and Sanchez-Cespedes J (2007) Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. J Antimicrob Chemother 59: 1210-1215.
16. Opazo A, Mella S, Dominguez M, Bello H, Gonzalez G (2009) Bombas de expulsión multidrogas en *Acinetobacter baumannii* y resistencia a antimicrobianos. Rev Chil Infect 26: 499-503.
17. Luo L, Jiang X, Wu Q, Wei L, Li J, Ying C (2011) Efflux pump overexpression in conjunction with alteration of outer membrane protein may induce *Acinetobacter baumannii* resistant to imipenem. Chemotherapy 57: 77-84.
18. Poirel L, Naas T, Nordmann P (2010) Diversity, epidemiology, and genetics of class D β-lactamases. Antimicrob Agents Chemother 54: 24-38.
19. Walther-Rasmussen J and Hoiby N (2006) OXA-type carbapenemases. J Antimicrob Chemother 57: 373-383.
20. Jacoby G and Bush K (2011) http://www.lahey.org/Studies/other.asp#table1 , accessed on 22 November 22 2011.
21. Paton R, Miles RS, Hood J, Amyes SGB (1993) ARI-1: β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. Int J Antimicrob Agents 2: 81-88.
22. Donald HM, Scaife W, Amyes SGB, Young HK (2000) Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. Antimicrob Agents Chemother 44: 196-199.
23. Scaife W, Young HK, Paton RH, Amyes SGB. (1995) Transferable imipenem resistance in *Acinetobacter* species from a clinical source. J Antimicrob Chemother 36: 585-586.
24. Higgins P, Poirel L, Lehmann M, Nordmann P, Seifert H (2009) OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in *Acinetobacter baumannii*. Antimicrob Agents Chemother 53: 5035-5038.
25. Higgins P, Dammhayn C, Hackel M, Seifert H (2010) Global spread of carbapenem-resistant *Acinetobacter baumannii*. J Antimicrob Chemother 65: 233-238.
26. Evans BA, Hamouda A, Towner KJ, Amyes SGB (2008) OXA-51-like beta-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*. Clin Microbiol Infect 14: 268-275.
27. Bonnet R, Marchandin H, Chanal C, Sirot D, Labia R, De Champs C, Jumas-Bilak E, Sirot J (2002) Chromosome-encoded class D β-lactamase OXA-23 in *Proteus mirabilis*. Antimicrob Agents Chemother 46: 2004-2006.
28. Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P (2008) *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. Antimicrob Agents Chemother 52: 1252-1256.

29. Koh TH, Sng L, Yeng Wang G, Hsu L, Zhao Y (2007) IMP-4 and OXA β-lactamases in *Acinetobacter baumannii* from Singapore. J Antimicrob Chemother 59: 627-632.
30. Poirel L, Mansour W, Bouallegue O, Nordmann (2008)a Carbapenem-resistant *Acinetobacter baumannii* isolates from Tunisia producing the OXA-58-like carbapenem-hydrolysing oxacillinase OXA-97. Antimicrob Agents Chemother 52: 1613-1617.
31. Afzal-Shah M, Woodford N, Livermore D (2001) Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother 45: 583-588.
32. Lu PL, Doumith M, Livermore D, Chen TP, Woodford N (2009) Diversity of carbapenem resistance mechanisms in *Acinetobacter baumannii* from Taiwan hospital: spread of plasmid-borne OXA-72 carbapenemase. J Antimicrob Chemother 63: 641-647.
33. Poirel L and Nordmann P (2006) Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. Antimicrob Agents Chemother 50: 1442-1448.
34. Villegas M, Kattan N, Correa A, Lolans K, Guzman A, Woodford N, Livermore D, Quinn J and the Colombian Nosocomial Bacterial Resistance Study Group (2007) Dissemination of *Acinetobacter baumannii* clones with OXA-23 Carbapenemase in Colombian hospitals. Antimicrob Agents Chemother 51: 2001-2004.
35. Merkier A, Catalano M, Ramirez MS, Quiroga C, Orman B, Ratier L, Famiglietti A, Vay C, Di Martino A, Kaufman S, Centron D (2008) Polyclonal spread of bla_{OXA-23} and bla_{OXA-58} in *Acinetobacter baumannii* isolates from Argentina. J Infect Dev Ctries 2: 235-240.
36. Carvalho KR, D'A Carvalho-Assef AP, Peirano G, Dos Santos LCG, Pereira MJF, Asensi MD (2009) Dissemination of multidrug-resistant *Acinetobacter baumannii* genotypes carrying bla_{OXA-23} collected from hospitals in Rio de Janeiro, Brazil. Int J Antimicrob Agents 34: 25-28.
37. Schimith Bier KE, Luiz SO, Scheffer MC, Gales AC, Paganini MC, Nascimento AJ, Carignano E, Dalla Costa LM (2010) Temporal evolution of carbapenem-resistant *Acinetobacter baumannii* in Curitiba, southern Brazil. Am J Infect Control 38: 308-314.
38. Fernández Colón E, Bustamante García Zu, Zamora Baldemama J, Zahalaga Via S, Pinto Davalos J, Funes Espinoza F Sevillano Peña E, Umaran Sánchez A, Gallego Andrés L (2009) Determinación de carbapenemasas y su relación con estructuras genéticas en aislamientos clínicos de *Acinetobacter baumannii* de hospitales de la ciudad de Cochabamba. BIOFARBO 17: 30-38.
39. Salazar E, Nieves B, Ruiz M, Ruiz J, Vila J, María A, Elsa V (2007) Molecular epidemiology and characterization of resistance mechanisms to various antimicrobial agents in *Acinetobacter baumannii* isolated in Mérida, Venezuela. Med Sci Monit 13: BR89-BR94.
40. Marque S, Poirel L, Heritier C, Brisse S, Blasco MD, Filip R, Coman G, Naas T, Nordmann T (2005) Regional occurrence of plasmid-mediated carbapenem-hydrolyzing oxacillinase OXA-58 in *Acinetobacter* spp. in Europe. J Clin Microbiol 43: 4885-4888.
41. Marti S, Sanchez-Céspedes J, Blasco MD, Ruiz M, Espinal P, Alba V, Fernández-Cuenca Felipe, Pascual A. and Vila J (2008) Characterization of the carbapenem-hydrolyzing Oxacillinase OXA-58 in an *Acinetobacter* Genospecies 3 clinical isolate. Antimicrob Agents Chemother 52: 2955-2958.
42. Werneck JS, Picao RC, Carvalhaes CG, Cardoso JP, Gales AC (2010) OXA-72-producing *Acinetobacter baumannii* in Brazil: a case report. J Antimicrob Chemother 66: 452-454.
43. Barnaud G, Zihoune N, Richard JD, Hippeaux MC, Eveillard M, Dreyfuss D, Branger C (2010) Two sequential outbreaks caused by multidrug-resistant *Acinetobacter baumannii* isolates producing OXA-58 or OXA-72 oxacillinase in an intensive care unit in France. J Hosp Infect 76: 358-360.
44. Goic-Barisic I, Towner K, Kovacic A, Sisko-Kraljevic K, Tonkic M, Novak A, Punda-Polic V (2011) Outbreak in Croatia caused by a new carbapenem-resistant clone of *Acinetobacter baumannii* producing OXA-72 carbapenemase. J Hosp Infect 77: 368-369.
45. Opazo-Capurro A, Bello H, Dominguez M and González-Rocha G (2010) First detection of $bla_{OXA-24-like}$ gene in *Acinetobacter baumannii* isolated from a Chilean Hospital. Abstract Book 8th International Symposium on the Biology of Acinetobacter, 1- 3 September 2010, Rome, Italy pp 86
46. Brown S, Young HK, Amyes SGB (2005) Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. Clin Microbiol Infect 11: 15-23.
47. Evans BA, Hamouda A, Amyes SGB (2007) OXA-type β-lactamases in *Acinetobacter baumannii*: emerging from the shadow of the extended-spectrum β-lactamases. Rev Med Microbiol 18: 63-72.
48. Brown S, Bantar C, Young HK, Amyes SGB (1998) Limitation of *Acinetobacter baumannii* treatment by plasmid-mediated carbapenemase ARI-2. The Lancet 17: 186-187.
49. Dalla Costa, L.; Coelho, J.; Souza, H.; Castro, M.; Stier, C.; Bragagnolo, K.; Rea-Neto, A.; Penteado-Filho, S.; Livermore, D.; Woodford, N. (2002). Abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy. 42: 119

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Conflict of Interests: No conflict of interests is declared.

J Antimicrob Chemother 2012; **67**: 1619–1622
doi:10.1093/jac/dks087 Advance Access publication 14 March 2012

Journal of Antimicrobial Chemotherapy

Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates

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Received 5 December 2011; returned 31 December 2011; revised 15 February 2012; accepted 24 February 2012

Objectives: To investigate the mechanism of ceftazidime resistance in two isogenic *Acinetobacter baumannii* strains from the United Arab Emirates.

Methods: Two *A. baumannii* strains, NM55 and NM128, were isolated 4 months apart from a 6-year-old patient in the United Arab Emirates. Genotypic characterization was performed by PFGE and the MIC of ceftazidime was determined by the agar dilution method. Detection of bla_{OXA} and metallo-β-lactamase genes was performed by multiplex PCR. Analysis of bla_{PER-7}, IS*Aba1*, bla_{ADC} and the IS*CR1* element was carried out by standard PCR. Plasmid analysis was achieved by Southern blotting.

Results: Strain NM55 was resistant to ceftazidime, whereas strain NM128 was susceptible. Both isolates carried the bla_{OXA-23} and bla_{OXA-64} genes and were identical according to their PFGE patterns. IS*Aba1* was present upstream of the bla_{OXA-23} gene, but absent upstream of bla_{ADC-26}, in both strains. Strain NM55 possessed a bla_{PER-7} gene with the presence of *gst*, a fragment of the *abc* transporter and a transposase gene downstream of it. The entire structure was part of an IS*CR1* element and was located on an ~200 kb plasmid in strain NM55, while the ceftazidime-susceptible NM128 strain carried an ~180 kb plasmid without the bla_{PER-7} gene.

Conclusions: Ceftazidime resistance was mediated by a PER-7 β-lactamase encoded in an IS*CR1* element located on a plasmid. This represents the first detection of a PER-7 β-lactamase encoded by a plasmid in *A. baumannii*.

Keywords: IS*CR1* element, Middle East, plasmid-borne bla_{PER-7}

Introduction

Acinetobacter baumannii is an opportunistic pathogen frequently causing outbreaks in intensive care units.[1] The proportion of multidrug-resistant *A. baumannii* isolates has risen recently and is considered as a global sentinel event.[1]

Cephalosporin resistance in *A. baumannii* almost invariably results from the overexpression of a family of AmpC β-lactamases, called ADC (*Acinetobacter*-derived cephalosporinases),[2] controlled by IS*Aba1* upstream of the gene providing a strong promoter.[3] Recently, a new variant of the Ambler class A PER-type β-lactamases, PER-7, with a broad activity against cephalosporins has been identified in *A. baumannii*. It was chromosomally encoded and associated with a mosaic class 1 integron structure, belonging to the IS*CR1* family.[3] Structurally, PER-7 has four amino acid substitutions compared with PER-1 and one compared with PER-6.[3] The aim of this study was to identify the gene(s) responsible for ceftazidime resistance in the non-susceptible member of the pair of isogenic strains of *A. baumannii* isolated in the United Arab Emirates and to characterize its genetic environment.

Materials and methods

Bacterial strains and PFGE

Two strains of multidrug-resistant *A. baumannii* were isolated in May (NM55) and August (NM128) 2008 from tracheal aspirates of a 6-year-old patient in Tawam Hospital, Al Ain, United Arab Emirates. Species identification was confirmed by PCR detecting the $bla_{OXA-51-like}$ gene[4] and by sequencing of a 455 bp section of the *rpoB* gene.[5]

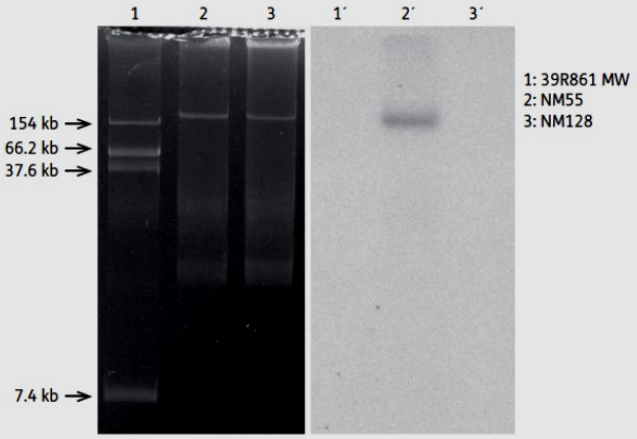

Figure 1. Southern blot of the plasmids extracted from *A. baumannii* strains NM55 and NM128 hybridized with a bla_{PER-7} probe. The gel on the left shows the plasmid profiles while the corresponding hybridization is shown on the right.

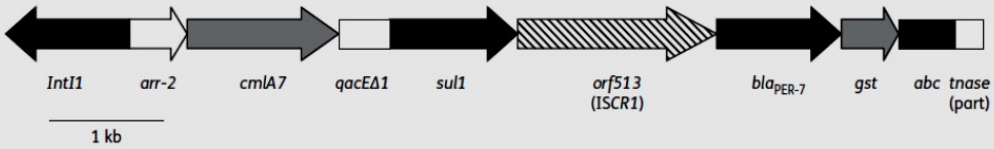

Figure 2. Schematic representation of the genetic environment of the bla_{PER-7} gene. The genetic orientations are represented by horizontal arrows. Partial genes are represented by squares. *tnase*, transposase.

Genomic DNA was digested with ApaI and the fragments were separated using a CHEF-DRII system (Bio-Rad, Hercules, CA, USA).

Susceptibility testing

Antibiotic susceptibility tests were performed by the disc diffusion method according to BSAC guidelines. The antibiotics tested were imipenem, meropenem, cefotetan, rifampicin, aztreonam, chloramphenicol, rifampicin, cefoperazone, cefepime, cefotaxime and cefpodoxime. The ceftazidime and rifampicin MICs were determined according to BSAC recommendations.

Detection of β-lactamase genes and IS elements

The detection and sequencing of $bla_{ADC-like}$ genes were performed by PCR using the primers ADC-F (5′-GCGCCGTGAATTCTTAAGTG-3′) and ADC-R (5′-CAGCTTATGCTGTGCTGGAT-3′), which align outside the $bla_{ADC-like}$ genes, detecting any genetic element upstream. The detection of OXA-type carbapenemases and metallo-β-lactamases was achieved using a multiplex PCR assay.[6,7] The presence of the $bla_{VEB-like}$, $bla_{GES-like}$, $bla_{NDM-like}$ and $bla_{PER-like}$ genes was determined by standard PCR using the primers VEB-F (5′-ATTTCCCGATGCAAAGCGT-3′), VEB-R (5′-CCAACAGC GATGAACAAACT-3′), GES-F (5′-ATGCGCTTCATTCACGCAC-3′), GES-R (5′-AA CTCATCCTGAGCACGGAC-3′), PER-F (5′-CCTGACGATCTGGAACCTTT-3′), PER-R (5′-GCAACCTGCGCAATGATAGC-3′), NDM-F (5′-GGGCCGTATGAGTGA TTG-3′) and NDM-R (5′-GCACACTTCCTATCTCGAC-3′). The presence of ISA*ba1* upstream of the $bla_{OXA-23-like}$ gene was identified using a combination of the ISA*ba1*-F (5′-CATTGGCATTAAACTGAGGAGAAA-3′) and OXA-23-R (5′-TCACAACAACTAAAAGCACTG-3′) primers. Sequencing of the $bla_{OXA-51-like}$ gene and the detection of ISA*ba1* upstream were performed according to Evans *et al.*[8]

Characterization of the genetic environment of bla_{PER-7}

The detection and characterization of the class 1 integron were performed by standard PCR,[9] using different combinations of primers. The detection of *orf513* was carried out by PCR using the ORF513-F (5′-TCAAAGAGACGACTCTGTGATGGAT-3′) and ORF513-R (5′-TGACTCTTAT CCAACGCTTTGGC-3′) primers. In order to investigate the genetic environment and sequence of the $bla_{PER-like}$ gene, standard PCR assays and sequencing were performed using the following combinations of primers: ORF513-F and PER-R, PER-F and gst-R (5′-GTTCAGTGGCTTCCCCTTTT-3′), and gst-F (5′-GAACGGCCTTCAGACTCAAA-3′) and Abau-R (5′-GGGTTTCC GAGAAGGTGATT-3′).

Plasmid analysis

Plasmids were extracted using a standard protocol and transferred to Hybond N+ membranes (GE Healthcare, UK). The hybridization and detection were carried out using the DIG DNA Labeling and Detection Kit (Roche Applied Sciences). Accurate sizing of the plasmid was carried out by PFGE against standard markers following S1 nuclease digestion.

Results

Both strains were identified as *A. baumannii* through the detection of the $bla_{OXA-51-like}$ and *rpoB* genes. The isolates were considered isogenic as they were isolated consecutively from the same patient 4 months apart and exhibited the same PFGE patterns. The strains were resistant to imipenem, meropenem, cefotetan, aztreonam, cefoperazone, cefepime, cefotaxime and cefpodoxime. However, strain NM55 was also resistant to ceftazidime and rifampicin whereas strain NM128 was susceptible to both of these antibiotics. The MICs of ceftazidime were 64.0 mg/L for isolate NM55 and <0.2 mg/L for isolate NM128. Moreover, the MICs of rifampicin were 32.0 mg/L for isolate NM55 and 2.0 mg/L for isolate NM128.

Both strains harboured the bla_{ADC-26} gene, but neither of them possessed any insertion elements upstream of the gene, suggesting that this gene was not overexpressed.

The bla_{OXA-23} gene was associated with IS*Aba1* upstream in both isolates, which can account for their resistance to carbapenems. The most notable difference between the strains was that only the ceftazidime-resistant NM55 gave a positive reaction when tested with the bla_{PER}-specific PCR. Through sequencing, the $bla_{PER-like}$ gene was identified as bla_{PER-7} (GenBank accession no. AEI54993.1).

Both strains harboured a large plasmid (Figure 1). The plasmid sizes were determined by S1 nuclease digestion, which showed that NM55 harboured an ~200 kb plasmid whereas NM128 carried an ~180 kb plasmid (sizing figure not shown). By Southern blotting (Figure 1), we determined that the bla_{PER-7} gene was located on the large plasmid of NM55, but not on the large plasmid of NM128. No conjugal transfer of the bla_{PER-7}-containing plasmid was achieved, irrespective of the recipient used.

The bla_{PER-7} gene was located within a complex class 1 integron, also containing the *arr-2* and *cmlA7* genes in the variable zone of the classic integron class 1 element (Figure 2), which confer resistance to rifampicin and chloramphenicol, respectively. The 3′-CS region of the class 1 integron contained the *qacEΔ1* and *sul1* genes, but the *orf5* gene was absent. Downstream of bla_{PER-7} was located a *gst* gene. Interestingly, further downstream there was part of an *abc* transporter gene (GenBank accession no. CP001172.1) and then a transposase gene (GenBank accession no. CP000863.1) (Figure 2).

Discussion

Here we report on the isolation of a bla_{PER-7}-carrying, ceftazidime-resistant *A. baumannii* (NM55) from the Middle East, the first such isolate from the region. Unlike the French strain AP2,[3] the gene in NM55 was located on an ~200 kb plasmid, although also without an apparent insertion sequence (IS) element upstream to provide a surrogate promoter. The results of the hybridization analysis support the hypothesis of the plasmid location of bla_{PER-7}. The bla_{ADC-26} gene was detected in both strains; it is not an extended-spectrum AmpC cephalosporinase (ESAC) gene and lacked an IS element upstream providing a promoter, indicating ceftazidime resistance was not due the activity of this enzyme. The genetic environment of the plasmid-borne bla_{PER-7} gene in strain NM55 differed (GenBank accession no. JQ639792), specifically in the downstream region, from the chromosomally located allele in the French isolate AP2: in the French isolate the chromosomal gene *ABAYE3396* was located downstream, while we found both *gst* and a part of an *abc* transporter gene. This genetic environment is similar to that described by Xia *et al.*[10] in *Aeromonas punctata* isolated in China, which harboured a bla_{PER-1} gene inserted in an *ISCR1* element. Further downstream, a transposase gene was located, interrupting the *abc* transporter gene. This indicates a probable insertion of the *ISCR1* element in this locus, which may represent a hot spot for the integration of mobile genetic elements. The strain NM55 was less susceptible to rifampicin in comparison with strain NM128, probably due the presence of the *arr-2* gene in the variable zone of the classic class 1 integron; however, both strains were resistant to chloramphenicol, suggesting that the mechanism of resistance was not caused only by the presence of the *cmlA7* gene in the variable zone.

On the other hand, there are also similarities between the French isolate AP2 and NM55. Both had the same $bla_{OXA-51-like}$ gene, namely bla_{OXA-64}, which is one of the most common genes in strains from the Middle East (A. Al Hasan, L. Al Hassan and S. G. B. Amyes, unpublished results). It is noteworthy that the French strain was isolated in 2010 whereas strain NM55 was isolated nearly 2 years earlier. This chronology may suggest that the PER-7 β-lactamase was originally carried into strains of *A. baumannii* on plasmids, such as the one described here, and some of the integrons migrated onto the chromosome. As in our study the plasmid was non-conjugative, the mechanism of its uptake is still to be determined.

This study shows the remarkable ability of *A. baumannii* to capture and/or lose genetic structures, which may be mediated by novel genetic structures, such as *ISCR1*, affecting their susceptibility to antibiotics that represent important therapeutic options. However, the strain had lost the bla_{PER-7} gene some 4 months after its first isolation, leaving the resultant strain, NM128, vulnerable to ceftazidime. The reasons for this loss are difficult to explain in an environment where cephalosporins are being used and it is a rare example of spontaneous resistance loss in *A. baumannii*.

The two isolates came from the same patient and had the same PFGE pattern, suggesting that the smaller plasmid of NM128 was a derivative of the plasmid of NM55, which had undergone a deletion, including bla_{PER-7}. Further detailed analysis is underway to confirm the exact mechanism by which this may have occurred.

Acknowledgements

The cooperation of the staff of the Microbiology Laboratory, Tawam Hospital, Al Ain, United Arab Emirates in collecting these isolates is highly appreciated.

Funding

This work was supported by the Chilean National Commission for Scientific and Technological Research (CONICYT), FMHS UAEU grant NP 10/07 and UAEU grant 01-10-8-11/09.

Transparency declarations

None to declare.

References

1 Peleg AY, Seifert H, Paterson D. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* 2008; **21**: 538–82.

2 Rodriguez-Martinez JM, Nordmann P, Ronco E *et al*. Extended-spectrum cephalosporinase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2010; **54**: 3484–8.

3 Bonnin R, Potron A, Poirel L *et al*. PER-7, an extended-spectrum β-lactamase with increased activity toward broad-spectrum cephalosporins in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2011; **55**: 2424–7.

4 Hamouda A, Evans BA, Towner K *et al*. Characterization of epidemiologically unrelated *Acinetobacter baumannii* isolates from four continents by use of multilocus sequence typing, pulsed-field gel electrophoresis, and sequence-based typing of $bla_{\text{OXA-51-like}}$ genes. *J Clin Microbiol* 2010; **48**: 2476–83.

5 Gundi VA, Dijkshoorn L, Burignat S *et al*. Validation of partial *rpoB* gene sequence analysis for the identification of clinically important and emerging *Acinetobacter* species. *Microbiology* 2009; **155**: 2333–41.

6 Woodford N, Ellington M, Coelho J *et al*. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents* 2006; **27**: 351–3.

7 Ellington MJ, Kistler J, Livermore D *et al*. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *J Antimicrob Chemother* 2007; **59**: 321–2.

8 Evans BA, Hamouda A, Towner K *et al*. OXA-51-like β-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2008; **14**: 268–75.

9 Bae IK, Lee Y, Lee W *et al*. Novel complex class 1 integron bearing an I*SCR1* element in an *Escherichia coli* isolate carrying the $bla_{\text{CTX-M-14}}$ gene. *Antimicrob Agents Chemother* 2007; **51**: 3017–9.

10 Xia R, Guo X, Zhang Y *et al*. *qnrVC*-like gene located in a novel complex class 1 integron harbouring the I*SCR1* element in an *Aeromonas punctata* strain from an aquatic environment in Shandong province, China. *Antimicrob Agents Chemother* 2010; **54**: 3471–4.

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ANTAGE-3761; No. of Pages 2

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International Journal of Antimicrobial Agents xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Letter to the Editor

The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary

Sir,

The increase in carbapenem resistance in *Acinetobacter baumannii* is largely attributable to the Ambler class D β-lactamases, in particular enzymes related to OXA-23 and OXA-58. The purpose of this study was to analyse the resistance in *A. baumannii* strains isolated from Aberdeen Royal Infirmary (Aberdeen, UK) from 2006 to 2010.

Nine non-repetitive *A. baumannii* strains were chosen for this study. The strains were identified by polymerase chain reaction (PCR) of the $bla_{\text{OXA-51-like}}$ gene and by sequencing of the *rpoB* gene. Minimum inhibitory concentrations (MICs) were determined according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC). The $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-143-like}}$ gene families were screened by multiplex PCR [1]. ISADC1 and OXA-23R primers were used for the detection of IS*Aba1* upstream of the $bla_{\text{OXA-23-like}}$ gene [1,2]. Aminoglycoside resistance genes were identified by multiplex PCR [3]. Primers for amplification of the bla_{ADC} gene have been described previously [2], and primers FU (5′-GCG CCG TGA ATT CTT AAG TG-3′) and RU (5′-AGC CAT ACC TGG CAC ATC AT-3′) were used to amplify the intergenic region upstream of the bla_{ADC} gene. PCR was performed for amplification of the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes [4], and gene fragments were sequenced for determining specific amino acid changes. PCR for detection of the class 1 integrase gene was performed as described previously [4].

A macrorestriction assay followed by pulsed-field gel electrophoresis (PFGE) was performed on all *A. baumannii* strains. Cluster analysis was performed by the unweighted pair-group method with mathematical averaging (UPGMA), and DNA relatedness was calculated using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimisation setting for the whole profile. Gel analysis was performed using BioNumerics v2.5 software (Applied Maths, Sint-Martens-Latem, Belgium). A value of ≥80% was chosen as the threshold for the establishment of clonal relatedness of the isolates.

S1 nuclease (Promega, Southampton, UK) digestion using 10 U of enzyme with incubation at 37 °C for 45 min was performed for the PFGE plugs according to the manufacturer's instructions. Plasmid curing was performed using acriflavine and with an elevated temperature of incubation. The strains were serially subcultured for 14 days at 47 °C.

Two novel variants of the $bla_{\text{OXA-51-like}}$ gene were found (Table 1): strain 14 had serine-14 (TCT) of the $bla_{\text{OXA-180}}$ gene replaced by phenylalanine (TTT) and is now designated $bla_{\text{OXA-216}}$; and strain 6n had threonine-255 (ACA) of the $bla_{\text{OXA-78}}$ gene substituted by isoleucine (ATA) and is now designated $bla_{\text{OXA-217}}$. Isolates 10 and 10n had variants of $bla_{\text{OXA-65}}$ gene with synonymous mutations.

Strains 3 and 12 possessed the *aac(3′)-Ia* gene conferring gentamicin resistance and had high ceftazidime MICs owing to the presence of IS*Aba1* upstream of the bla_{ADC} gene. All of the remaining strains (except 10) had the bla_{ADC} gene without IS*Aba1* upstream and thus were susceptible to ceftazidime (Table 1). Strain 10 completely lacked the bla_{ADC} gene. All of the isolates were susceptible to colistin.

Strains 3 and 12 had amino acid changes at position 83 of GyrA (serine83 → leucine) and position 80 of ParC (serine80 → leucine) conferring ciprofloxacin resistance. Both strains possessed integrase genes and sequencing confirmed the presence of putative glucose dehydrogenase precursor that could be responsible for catabolism of glucose by oxidation.

The PFGE profiles of the strains showed that most were not clonally related as they had <80% similarity (data not shown). PCR for insertions causing disruption of *carO* (29 kDa outer membrane protein) was not detected for any of the strains. PFGE analysis revealed that strains 3 and 12 had 83% similarity; strain 3, isolated in the year 2006, was negative for the IS*Aba1*–$bla_{\text{OXA-23}}$ gene, whereas strain 12, isolated in the year 2008, was positive and was resistant to imipenem and meropenem (Table 1). Strains 3 and 12

Table 1
Resistance profiles of *Acinetobacter baumannii* clinical strains.

Strain no.	Source of isolation	Date of isolation	MIC (mg/L)						*int1*	bla_{ADC}	IS*Aba1*–bla_{ADC}	$bla_{\text{OXA-51-like}}$ gene
			MEM	IPM	CAZ	GEN	CIP	COL				
3	Blood	09/09/2006	1	0.5	64	256	32	0.25	+	+	+	66
10	Blood	08/06/2008	0.06	0.06	1	0.12	0.12	0.12	–	–	–	65 (variant)
12	Blood	25/08/2008	16	16	64	8	32	0.5	+	+	+	66
14	Blood	21/09/2009	0.06	0.06	4	0.03	0.12	0.03	–	+	–	216
16	Sputum	20/02/2010	0.5	0.5	4	0.12	0.12	0.12	–	+	–	51
3n	Blood	27/04/2006	0.5	0.25	2	0.03	0.12	0.12	–	+	–	64
6n	Blood	17/07/2007	1	0.5	8	0.12	0.5	0.5	–	+	–	217
10n	Blood	22/10/2008	0.06	0.06	1	0.03	0.12	0.12	–	+	–	65 (variant)
14n	Blood	14/09/2009	0.5	0.25	8	0.12	0.5	0.12	–	+	–	89

MIC, minimum inhibitory concentration; MEM, meropenem; IPM, imipenem; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; COL, colistin.

doi:10.1016/j.ijantimicag.2011.12.004

Please cite this article in press as: Lopes BS, et al. The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary. Int J Antimicrob Agents (2012), doi:10.1016/j.ijantimicag.2011.12.004

had an identical $bla_{OXA-51-like}$ allele 1 corresponding to sequence group 1. In addition, strain 12 had the bla_{OXA-23} clone 1 allele, which belongs to European clone II. This indicates the *A. baumannii* is actively acquiring resistance genes, probably through plasmid transfer, although *S1* nuclease digestion and plasmid extraction procedures did not detect any plasmids, and elimination studies with acriflavine did not remove the resistance determinants. This suggests that if bla_{OXA-23} was plasmid borne, it is now integrated in the host chromosome of strain 12 endowing it with a stable mechanism of carbapenem resistance.

These results show that the clinical situation in the hospital in Aberdeen is in a state of flux. New variant strains are emerging and, most importantly, a carbapenem-sensitive strain has become resistant through acquisition of the bla_{OXA-23} gene with an IS*Aba1* element upstream that carries a promoter allowing expression of the β-lactamase. The bla_{OXA-23} gene was first found in Scotland more than 20 years ago and it has remained the sole mechanism of carbapenem resistance until this point [5].

Nucleotide accession numbers

The $bla_{OXA-216}$ and $bla_{OXA-217}$ genes have been deposited in GenBank under the accession nos. FR865168 and JN603240, respectively.

Funding: University of Edinburgh Overseas Research Scholarship to BSL; MRC grant number RA0119.

Competing interests: None declared.

Ethical approval: Not required.

References

[1] Higgins PG, Lehmann M, Seifert H. Inclusion of OXA-143 primers in a multiplex polymerase chain reaction (PCR) for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 2010;35:305.

[2] Ruiz M, Marti S, Fernandez-Cuenca F, Pascual A, Vila J. Prevalence of IS*Aba1* in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. FEMS Microbiol Lett 2007;274:63–6.

[3] Noppe-Leclercq I, Wallet F, Haentjens S, Courcol R, Simonet M. PCR detection of aminoglycoside resistance genes: a rapid molecular typing method for *Acinetobacter baumannii*. Res Microbiol 1999;150:317–22.

[4] Golanbar GD, Lam CK, Chu YM, Cueva C, Tan SW, Silva I, et al. Phenotypic and molecular characterization of *Acinetobacter* clinical isolates obtained from inmates of California correctional facilities. J Clin Microbiol 2011;49:2121–31.

[5] Donald HM, Scaife W, Amyes SG, Young HK. Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. Antimicrob Agents Chemother 2000;44:196–9.

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21 October 2011

Please cite this article in press as: Lopes BS, et al. The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary. Int J Antimicrob Agents (2012), doi:10.1016/j.ijantimicag.2011.12.004

G Model
ANTAGE-4245; No. of Pages 2
ARTICLE IN PRESS
International Journal of Antimicrobial Agents xxx (2014) xxx–xxx
Contents lists available at ScienceDirect
International Journal of Antimicrobial Agents
journal homepage: http://www.elsevier.com/locate/ijantimicag

Letter to the Editor

Novel genetic structure harbouring bla_{PER-1} in ceftazidime-resistant *Acinetobacter baumannii* isolated from Kuwait

Sir,

Acinetobacter baumannii has emerged as one of the most important pathogens associated with hospital-acquired infections worldwide. The number of multidrug-resistant *A. baumannii* isolates has increased in the last few years, making it a difficult micro-organism to treat [1]. Cephalosporins such as ceftazidime represent an important option to control infections caused by this micro-organism [1]. The aim of this work was to analyse the mechanism of ceftazidime resistance in an isolate collected from a patient in Kuwait in 2011.

A. baumannii isolate Kw5 was obtained from a 75-year-old patient from Amiri Hospital (Kuwait City, Kuwait). Species identification was performed by sequencing of the *rpoB* gene and detection of the $bla_{OXA-51-like}$ gene. Genotyping was achieved by multilocus sequence typing (MLST), and initial susceptibility tests were carried out by the disk diffusion method [2,3]. Minimum inhibitory concentrations (MICs) were determined according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC). The presence of extended-spectrum β-lactamase (ESBL) and carbapenem-hydrolysing class D β-lactamase (CHDL) genes, detection of the $bla_{ADC-like}$ gene, its association with insertion sequence IS*Aba1*, and determination of the sequence of the $bla_{PER-like}$ gene were performed by PCR and sequencing [3]. To study the contribution of $bla_{PER-like}$ and bla_{ADC} genes to ceftazidime resistance, the disk diffusion test was repeated with the addition of 500 μg of phenylboronic acid to the ceftazidime disk. Furthermore, the presence of metallo-β-lactamases (MBLs) was determined by the double-disk synergy test using ethylene diamine tetra-acetic acid (EDTA) as inhibitor.

The immediate genetic context of the bla_{PER-1} gene was characterised by inverse PCR using the invPER-F (5′-GCCGAACCAATGAAGCTATCATTGCGCAGG-3′) and invPER-R (5′-AATTTGCTCTTTTAACAGTGGGGATTGCGCTG-3′) primers. The PCR products obtained were sequenced and were then analysed by comparison with the sequences available in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids were extracted using a commercial kit following the manufacturer's instructions (Promega, UK) and were then extracted from the agarose gel using an extraction kit (QIAGEN, UK) to obtain pure plasmid DNA, which was then used as template for PCR to detect the bla_{PER-1} gene. Plasmid curing was attempted using 45 °C as the growing temperature. Conjugation by filter paper mating was performed to analyse the ability of the bla_{PER-1} gene to be mobilised between different isolates.

Kw5 strain was confirmed as *A. baumannii* through detection of the $bla_{OXA-51-like}$ gene and sequencing of the *rpoB* gene. The sequence type (ST) obtained corresponded to ST2, which belongs to the worldwide clone 2 (WW2) that has been previously detected in Kuwait [4]. Unlike Kw5 strain, the WW2 anteriorly described in the country harboured a GES-type β-lactamase conferring cephalosporin resistance [4]. The Kw5 strain was resistant to ampicillin, ampicillin/sulbactam, cefotaxime, cefepime, cefoxitin, piperacillin/tazobactam, ciprofloxacin, meropenem, imipenem and trimethoprim/sulfamethoxazole, whereas it was susceptible to tigecycline, amikacin, colistin and minocycline. Kw5 strain was highly resistant to ceftazidime, with a MIC of >256 mg/L. In addition, Kw5 was negative for all double-disk synergy tests using EDTA, which indicated the absence of MBLs.

The Kw5 isolate was positive for the $bla_{OXA-23-like}$ gene, which explains its resistance to carbapenems. An IS*Aba1* element was detected upstream of the $bla_{ADC-like}$ gene in Kw5, which has previously been described as the most common mechanism of ceftazidime resistance. Nevertheless, it was likely not to be the exclusive mechanism of resistance to this antibiotic, as the ESBL PER-1 was also detected. Its gene was located on a plasmid of ca. 140 kb. Plasmid curing using temperature as a curing agent failed even after 30 days exposure, indicating a high stability of this element; plasmid conjugation experiments were also unsuccessful.

The disk diffusion test using 500 μg of phenylboronic acid, a potent inhibitor of ADC β-lactamases, did not show differences between the inhibition zones, indicating that the ceftazidime resistance in this strain was likely to result from the activity of PER-1 β-lactamase. The bla_{PER-1} gene has been previously detected in *A. baumannii* associated with IS*Pa12* upstream, which increases its expression, and bracketed downstream by IS*Pa13* [5], comprising the transposon Tn*1213*. However, the genetic environment of bla_{PER-1} in Kw5 was different as there were copies of the IS*Pa12* gene flanking either side of the ESBL gene with a spacer region of 154 bp at both extremities (accession no. KF978125). These may help the bla_{PER-1} gene to mobilise between replicons, as the plasmid that harbours this structure could not be transferred by conjugation. The integrity of this complete transposon ensures that the potential of dissemination of this genetic structure is high; this is indicated by its location on a plasmid and the transposon-like structure should be self-mobilisable through the activity of the transposase of IS*Pa12*. This study has reconfirmed the remarkable ability of *A. baumannii* to capture and express antibiotic resistance genes, which is one of the most important factors making this micro-organism a persistent and dangerous nosocomial pathogen.

Nucleotide accession no.: The sequence of the transposon-like structure described in this work has been deposited in GenBank under the accession no. KF978125.

Funding: The authors wish to thank Kuwait University Research Administration and the Kuwait Foundation for Advancement of Science (KFAS), Kuwait, for funding the project [grant nos. NM02/10 and 2011130204, respectively]. The authors would

http://dx.doi.org/10.1016/j.ijantimicag.2014.01.001

Please cite this article in press as: Opazo A, et al. Novel genetic structure harbouring bla_{PER-1} in ceftazidime-resistant *Acinetobacter baumannii* isolated from Kuwait. Int J Antimicrob Agents (2014), http://dx.doi.org/10.1016/j.ijantimicag.2014.01.001

also like to acknowledge the Research Unit for Genomics, Proteomics and Cellomics Studies (OMICS) of the Health Sciences Centre, Kuwait University [Project No. SRUL02/13] for their help in performing DNA sequencing for this project, and the Chilean National Commission for Scientific and Technological Research (CONICYT).

Competing interests: None declared.

Ethical approval: Not required.

References

[1] Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev 2008;21:538–82.
[2] Nemec A, Krízová L, Maixnerová M, Diancourt L, van der Reijden TJK, Brisse S, et al. Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant strains of European clone II. J Antimicrob Chemother 2008;62:484–9.
[3] Opazo A, Sonnevend A, Lopes B, Hamouda A, Ghazawi A, Pal T, et al. Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates. J Antimicrob Chemother 2012;67:1619–22.
[4] Bonnin RA, Rotimi VO, Al Hubail M, Gasiorowski E, Al Sweih N, Nordmann P, et al. Wide dissemination of GES-type carbapenemases in *Acinetobacter baumannii* isolates in Kuwait. Antimicrob Agents Chemother 2013;57:183–8.
[5] Poirel L, Bonnin RA, Nordmann P. Genetic basis of antibiotic resistance in pathogenic *Acinetobacter* species. IUBMB Life 2011;63:1061–7.

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11 November 2013

Please cite this article in press as: Opazo A, et al. Novel genetic structure harbouring bla_{PER-1} in ceftazidime-resistant *Acinetobacter baumannii* isolated from Kuwait. Int J Antimicrob Agents (2014), http://dx.doi.org/10.1016/j.ijantimicag.2014.01.001

APPENDIX B:

Abstracts of conferences presentations

Ceftazidime resistance in *Acinetobacter baumannii* from the United Arab Emirates

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Acinetobacter baumannii has emerged as one of the most important microorganism causing infections in hospitalized patient. The only remaining options for the treatment of serious infectious diseases causes by multi-resistant *A. baumannii* are carbapenems, such as imipenem and meropenem; cephalosporins and colistin. Resistance to oxyiminocephalosporins (ceftazidime and cefotaxime) has been associated with the insertion sequence IS*Aba1* providing a strong promoter to ADC-genes.

3 strains of *A. baumannii* isolated in Al Ain, UAE, designated Nm55, Nm57 and Nm128, were studied. Nm57 was isolated from an intravenous catheter tip of an adult patient, Nm55 and Nm128 were both isolated from sputum samples taken 7 days apart from a 6 year old patient. They were confirmed as *A. baumannii* by PCR detecting the $bla_{\text{OXA-51-like}}$ gene and sequencing the *rpoB* gene. Genotypic characterization was performed by macro-restriction assay followed by pulsed-field gel electrophoresis (PFGE), using the *Apa*I enzyme. Antibiotic susceptibility was performed by disk diffusion test according to BSAC guidelines. Determination of minimal inhibitory concentration (MIC) of ceftazidime was performed by the agar dilution method according to BSAC recommendations. Detection of bla_{OXA} genes was performed by multiplex-PCR and detection of IS*Aba1* and ADC was done by standard-PCR using specific primers.

All the strains were designated as identical as they showed more than 94% similarity according to PFGE pattern and were all MLST type ST110. All three strains were resistant to imipenem, meropenem, cefotetan, aztreonam, cefoperazone, cefepime, cefotaxime and cefpodoxime. Strain Nm55 was resistant to ceftazidime, while Nm57 was intermediate and Nm128 was susceptible. All the strains harbour both OXA-64 chromosomal and the OXA-23 plasmid □-lactamases. IS*Aba1* was upstream of the $bla_{\text{OXA-23}}$ genes in all three strains and was responsible for carbapenem resistance. All the strains harboured the same bla_{ADC} gene but in no case was there a defined IS element upstream indicating that this gene was not being overexpressed.

This study shows a rare example of an *A. baumannii* strain that was resistant to carbapenems but was sensitive to ceftazidime. When the strain progressed to ceftazidime resistance this was not by the reported method of an insertion of an IS*Aba1*-like element upstream *bla*ADC gene providing the necessary promoter.

Conference: 51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). September, 17 – 20. Chicago, USA.

Detection of IS*26* in carbapenem non-susceptible *Acinetobacter baumannii*

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Objective

Analyse the role of IS*26* in the non-susceptible carbapenem phenotype of Ab-Ch5 strain isolated in Chile.

Methods

Species-identification was carried out by amplification of $bla_{\text{OXA-51-like}}$ gene and by sequencing of a 455bp section of the *rpo*B gene. Determination of the minimal inhibitory concentration (MIC) of carbapenems was performed by the agar double diffusion method. Detection of carbapenemase genes, insertion elements and the characterisation of the genetic environment of $bla_{\text{OXA-58}}$ were carried out by PCR. Plasmids were analysed by the use of commercial kits. Expression of $bla_{\text{OXA-58}}$ was determined by RT-PCR.

Results

MICs values of imipenem and meropenem were 8.0 mg/L and 4.0 mg/L, respectively. Strain Ab-Ch5 harboured both $bla_{\text{ADC-like}}$ and $bla_{\text{OXA-58}}$. The $bla_{\text{OXA-51-like}}$ variant was the $bla_{\text{OXA-51}}$ gene itself without the presence of IS*Aba1* element upstream. The characterisation of the genetic environment of $bla_{\text{OXA-58}}$ showed the presence of IS*26*-ΔIS*Aba3* arrangement upstream and an intact IS*Aba3*-element downstream. The $bla_{\text{OXA-58}}$ was detected in a plasmid of ca. 260 kb. There were not differences in the expression of $bla_{\text{OXA-58}}$ in comparison with an IS*Aba3*-$bla_{\text{OXA-58}}$ carbapenem-resistant strain.

Conclusion

The interruption of IS*Aba3* by the IS*26*-element upstream does not affect the control of the expression of the OXA-58 β-lactamase in strain Ab-Ch5. The presence of $bla_{\text{OXA-58}}$ in a plasmid represents a source of horizontal dissemination of this carbapenemase gene in which IS*26* may play an important role. This is the first report detecting the association of IS*26* with $bla_{\text{OXA-58}}$ in a strain isolated in South America.

Conference: 9th International Symposium on the Biology of *Acinetobacter*. June, 19 – 21. Cologne, Germany.